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14. ABSTRACT: Gene therapy holds great promise for treatment of breast cancer. In particular clinical trials are underway to apply therapeutic genes related to pro-drug activation or to modulate the activity of oncogenes by blocking promoter sites. However, there are major problems in terms of assessing the delivery to target tissue, assessing the uniformity (versus heterogeneity) of biodistribution and determining whether the genes are expressed. We propose to design, evaluate and apply a novel approach to gene activity detection- specifically, using fluorinated substrates of β-galactosidase to reveal gene activity. Our prototype molecule PFONPG (para- fluoro- ortho- nitro- phenyl β-Dgalactopyranoside) is a direct analog of the traditional "yellow' biochemical indicator ONPG (ortho- nitro- phenyl β-Dgalactopyranoside). This shows useful MR characteristics, sensitivity to enzyme activity and ability to enter cells. We will synthesize analogs of this prototype to optimize MR and biological characteristics and explore the feasibility of tailoring the reporter to specific applications, e.g., exploiting β-gal activity to deliver specific physiological reporter molecules such as pH and potentially specific cytotoxic agents. The agents will be rigorously tested in solution, applied to cultured breast cancer cells and ultimately used to examine β-gal activity in vivo in transfected breast tumors in mice and rats.

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Gene therapy, fluorine NMR, β-galactosidase, pH

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Introduction

Gene therapy holds great promise for treatment of breast cancer (1, 2). In particular, clinical trials are underway to apply therapeutic genes related to pro-drug activation or modulation of the activity of oncogenes by blocking promoter sites. However, there are major problems in terms of assessing the delivery to target tissue, assessing the uniformity (versus heterogeneity) of biodistribution, and determining whether the genes are expressed. We proposed to design, evaluate and apply a novel approach to gene activity detection- specifically, using fluorinated substrates of β-galactosidase to reveal gene activity. Our prototype molecule PFONPG (para- fluoro- ortho- nitro- phenyl β-D-galactopyranoside) is a direct analog of the traditional "yellow' biochemical indicator ONPG (ortho- nitro- phenyl β-D-galactopyranoside). This shows useful MR characteristics, sensitivity to enzyme activity and ability to enter cells. We will synthesize analogs of this prototype to optimize MR and biological characteristics and explore the feasibility of tailoring the reporter to specific applications, *e.g.*, exploiting β-gal activity to deliver specific physiological reporter molecules such as pH and potentially specific cytotoxic agents. The agents will be rigorously tested in solution, applied to cultured breast cancer cells, and ultimately used to examine β-gal activity *in vivo* in transfected breast tumors in mice and rats.

Statement of Work

Year 1

Task 1 Synthesize novel molecules to report activity of the β-galactosidase gene- minimum 8 novel agents (**Completed in Year 1**)

Task 2 Characterize novel agents (NMR, mass spec, colorimetric analysis) (**Completed in Year 1**)

Task 3 Test agents for enzyme activity in solution (**Completed in Year 1**)

Task 4 Test initial indicators in cultured breast cancer cells (control + transfected) (Months 6-12) (**Completed in Year 2**)

Year 2

Task 5 Scale up synthesis of most promising indicator for animal investigations (Months 13-15) (**Completed in Year 2**)

Task 6 Evaluate in mice (constitutively expressing β-gal ROSA animals); test dosing protocols, timing, MR detection protocols- (20 mice) (Months 13-18) (**Completed in Year 3**)

Task 7 Evaluate in rodents with stably transfected tumors and compare with traditional assays- (20 mice + 20 rats) (Months 15-24) (**Ongoing**)

Task 8 Synthesize second generation "smart" β-gal substrates as reporters of physical parameters such as pH or as cytotoxic agents (Months 15-24) (**Completed in Year 3**)

Year 3

Task 9 Apply optimal β-gal reporters to assess transfection efficiency, gene expression (spatial and temporal) in tumors *in vivo* (20 mice + 20 rats) (Months 25-34) (**Ongoing**)

Task 10 Evaluate "smart agents" *in vitro* (Months 25-34) (**Completed in Year 3**)

Task 11 Evaluate "smart agents" *in vivo* (Months 25-34) (**Ongoing)**

Task 12 Prepare manuscripts and final report (Months 34-36) (**Ongoing)**

Progress

While we have not completed all tasks within the original three year time table, we requested and were granted a 1 year no additional cost extension. I believe remaining tasks will be completed successfully.

Task 6 Completed during Year 3.

We found that administration of PFONPG (4-Fluoro-2-nitrophenol-β-D-galactopyranoside) to ROSA mice caused rapid death. We believe this is due to release of toxic aglycon (nitrophenol) causing rapid depolarization of the heart. Indeed, perfusion of a heart with the PFONP aglycon caused immediate cardiac arrest. However, we were able to show effective conversion of reporter molecules by tissues (heart, liver, and muscle) excised from ROSA mice, but not wild type mice. We are able to use the fluorophenylgalactopyranoside substrates administered systemically in normal mice without apparent toxicity. However, we are faced with competing wash-in and wash-out phenomena. In some cases, we have been able to detect signal from tumors following IP administration, but substrate signal was weak and washed out again. Thus, any conversion of substrate to algycon product by β-gal competes with substrate clearance. Likewise product clears, and thus, signal is difficult to detect.

We have now, however, established that direct intra tumoral injection of substrate provides excellent signal to noise (>10), which may be detected within 5 minutes by spectroscopy. Some substrate clearance occurs, but in β-gal expressing tumors, conversion to aglycon product is much faster and the product signal is readily detected. We have now conducted investigations in multiple mice expressing MCF7-lacZ or -WT tumors and the presence of β-gal is obvious. We have most recently examined paired tumor xenografts on opposite flanks of female mice. Both tumors could be examined simultaneously. As yet there has

been insufficient signal for imaging, but by exploiting pairs of reporter molecules as developed in Tasks 1-4, we are able to differentiate relative conversion of each of two substrates, simultaneously. LacZ tumors are readily identified by conversion of substrate to release aglycon product, whereas no conversion is seen in the wild type tumors (Fig. 1). As yet, we have compared stably transfected cells growing as tumors versus wild type, but during the next year, we will examine transient transfection efficiency *in situ* (Task 9). While our original goal was systemic IV or IP delivery of reporter molecules, direct intra tumoral injection represents considerable progress over the previous NMR approaches (3), which required intra cellular micro injection.

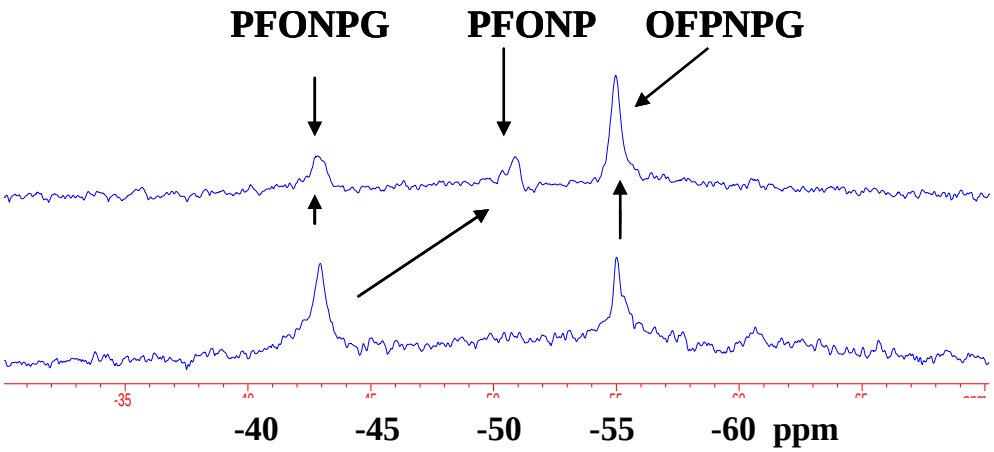

Figure 1

2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) and 4-Fluoro-2-nitrophenol-β-D-galactopyranoside (PFONPG) each have a single ^{19}F peak at -55 ppm and -43 ppm, respectively, relative to aqueous sodium trifluoroacetate (NaTFA). Following direct intra tumoral injection both substrates were observable in a spectrum observed after 10 mins (lower spectrum) (40 µl PFONPG in MCF7-lacZ tumor and 40 µl OFPNPG in wt-tumor with TR = 1 s and 5 ½ min per acquisition). Upon cleavage by β-gal over 1 hr, the aglycon PFONP was observed at a chemical shift of ~-51 ppm, but no OFPNP was generated at -60 ppm (upper spectrum).

Task 7 (ongoing)

Using investigations such as that shown in Figure 1, we have been able to identify breast tumors expressing lacZ (β-gal) *in vivo* and differentiate them from wild type. We have undertaken histological validation based on stains, Western blots, and enzyme activity following tumor excision (*e.g.*, Figure 2). We also generated a new MCF7-lacZ-luciferase cell line, which provides correlative studies by bioluminescent imaging of tumor location and extent.

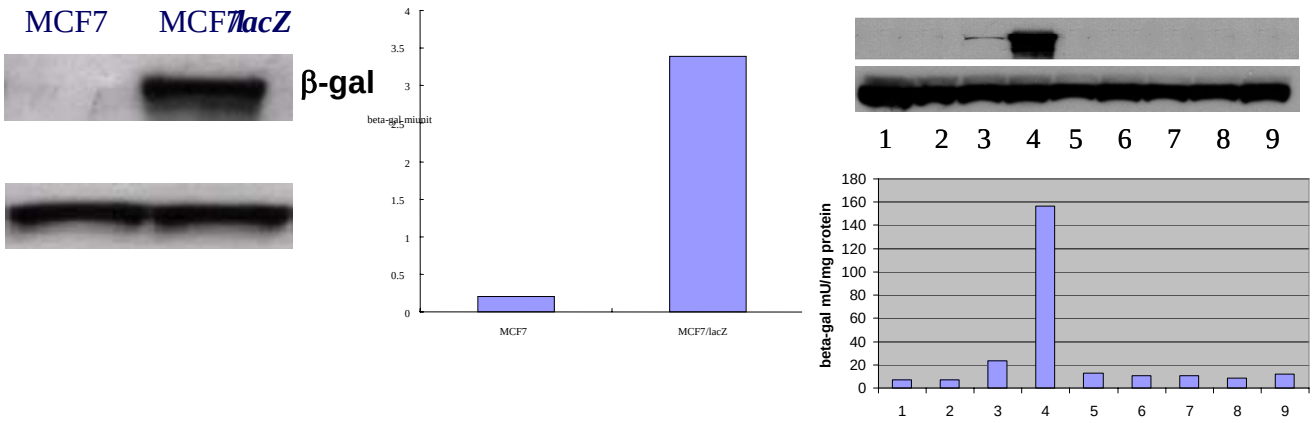

Figure 2 Verification of β-gal expression in tumors growing in mice using Western blot and enzyme activity analysis. Left and center: comparison of MCF7-WT and –LacZ tumors. Right Comparison with other mouse tissues 1. Liver; 2. Muscle; 3. MCF7-WT tumor; 4. MCF7-lacZ tumor; 5 Heart; 6. Spleen; 7. Lung; 8. Kidney; 9. Tail.

Task 8 Completed in year 3

Several "second" generation gene reporters were synthesized representing diverse classes of agents. I). GFPOL uses fluorinated vitamin B6 as the aglycone to reduce toxicity of product and results were published last year (4). GFPOL was indeed much less toxic, but also rather insensitive to

β-gal activity. Additional sugar moieties improved water solubility and response to β-gal and detection of the polyglyosylated agents have been published this year (appendix) (5). Results of enzyme activity and MCF7-lacZ cells are described under Task 10.

A problem with the ^{19}F NMR approach is that product aglycones are not trapped at site of activity, but washed out, and hence, difficult to detect. It occurred to us that fluoroaryl agents could be trapped by generating insoluble complexes as with the commercial black stain S-gal . A novel class of ^{19}F NMR *lacZ* gene reporter molecule based on fluorocatechols was designed (**LCD-1**), synthesized and tested (Figure 3).

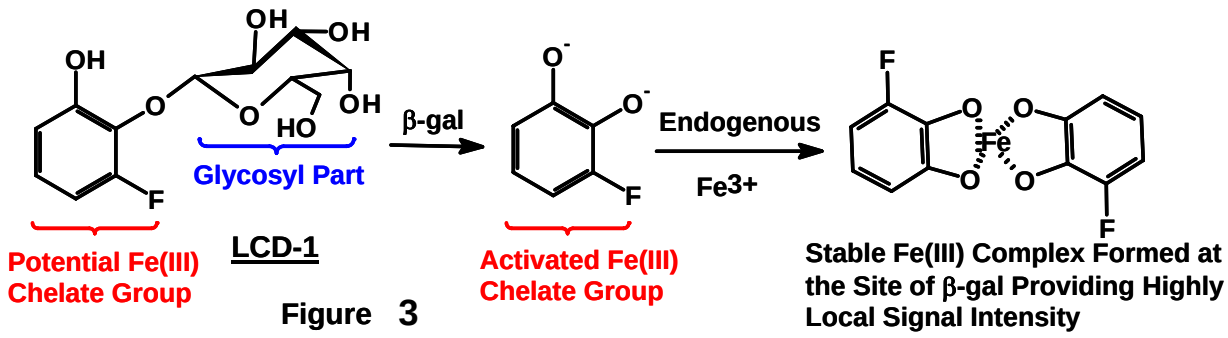

Figure 3

ii) In addition to seeking to reduce toxicity of reporter molecules, we have also sought to exploit enzyme activated release of toxic aglycons. We have achieved modest success (Fig. 4), but while lacZ cells showed greater toxicity the 100 uM concentrations are too high to provide effective activity *in vivo*.

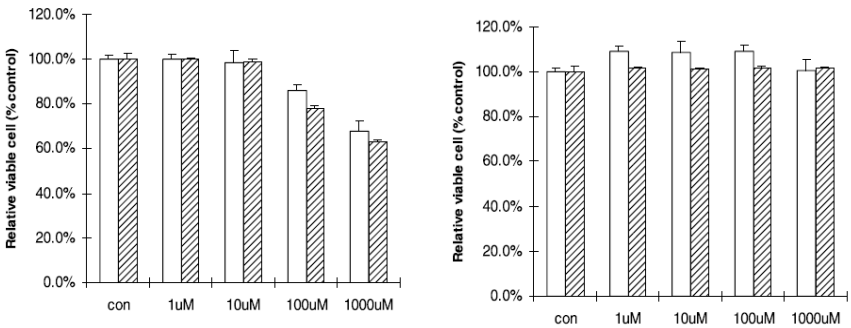

Figure 4. Cell viability of human MCF7 breast cancer cells in PBS (pH 7.4) with respect to exposure to

substrate 2-Nitro-4-trifluoromethylphenyl β-D-galactopyranoside left panel MCF7-lacZ cells; right panel: MCF7-WT. Open bars 48 h exposure; hatched bars 96 h exposure.

Task 9 Apply optimal β-gal reporters to assess transfection efficiency, gene expression (spatial and temporal) in tumors *in vivo* (Ongoing)

Task 10 Evaluate "smart agents" *in vitro* (Months 25-34)

Polyglycosylated FPOL substrates were added to β-gal enzyme of MCF7-lacZ cells. It became apparent that use of multiple galactose moieties was not appropriate since enzyme acted on each of them generating complex NMR spectra (Fig. 5). However, when secondary sugar moieties were glucose or mannose, there was selective enzyme activity (Fig. 6). We also determined the titration curves for each of the polyglyosylated substrates and products. (Table 1).

LCD-1 was examined with MCF7-lacZ cells and showed conversion yielding two signals (Fig. 7). In the presence of ferric ions a purple gelatinous precipitate was formed, but there was still a single narrow ^{19}F NMR signal. These preliminary data demonstrate the feasibility of a novel approach to detecting β-gal activity, *i.e*., generating complexes to trap the released reporter molecule products. Reassuringly, the product signals remain narrow and detectable, and we believe this approach shows promise for developing ^{19}F NMR approaches to gene reporter molecules.

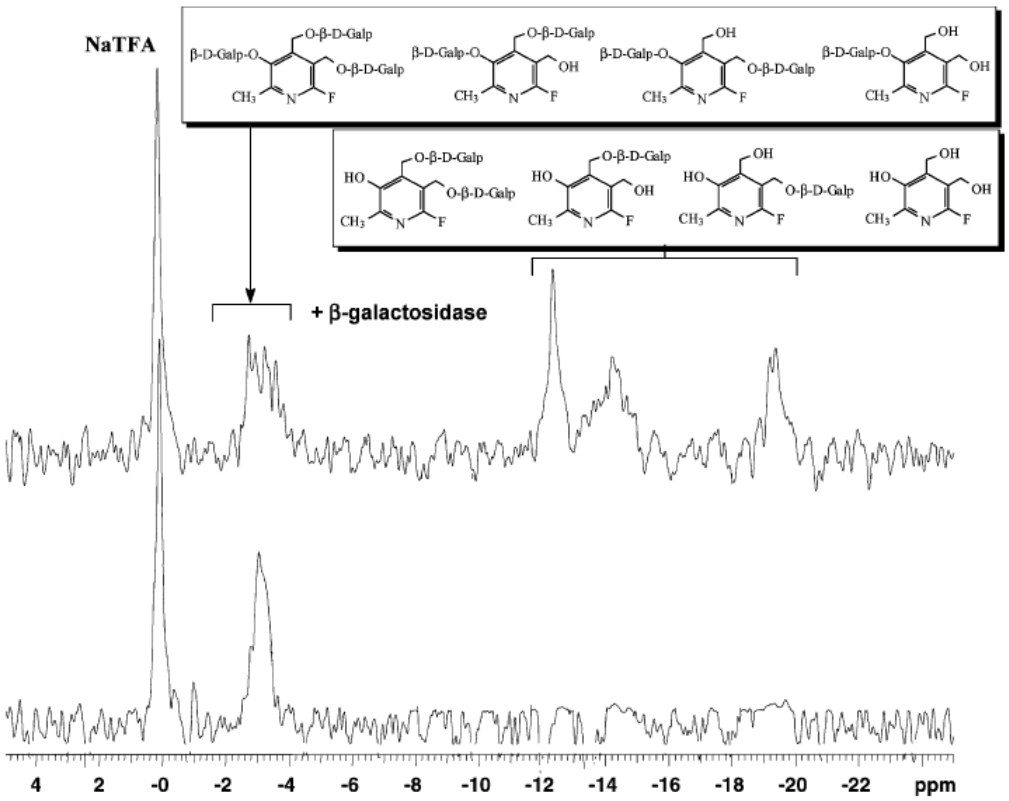

Figure 5. ^{19}F NMR spectra of 3,α^4, α^5-tri-*O*-(*β-D*-galactopyranosyl)-6-fluoropyridoxol (10.1 mg, 15 mmol, lower trace) and its products resulting from addition of *β*-gal (E801A, 15 units) in PBS (pH) 7.4) at 37 °C (upper trace). Spectra were acquired in 51 s and enhanced with an exponential line broadening 40 Hz; *β-D*-Galp) *β-D*-galactopyranosyl.

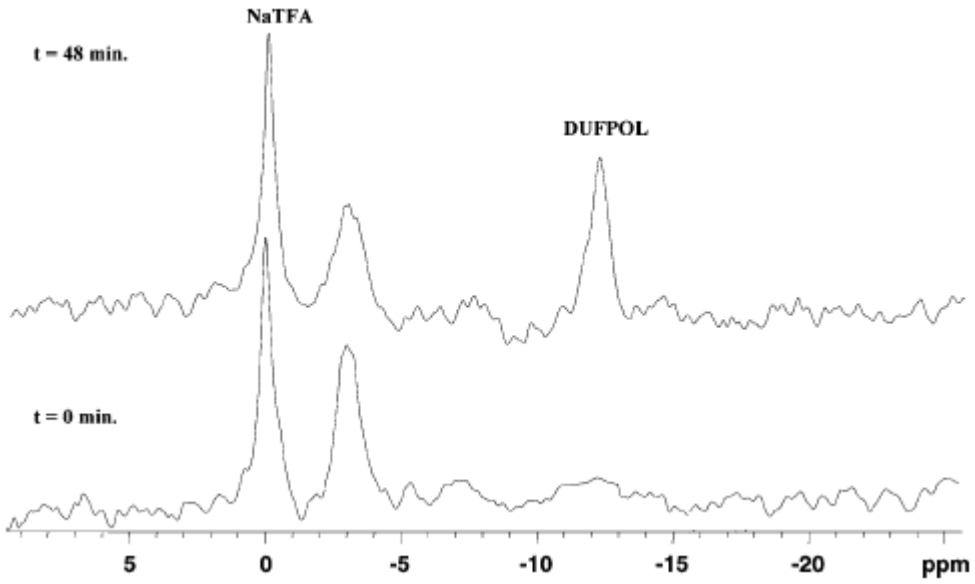

Figure 6. ^{19}F NMR spectra of 3-*O*-(*β*-D-galactopyranosyl)-α^4, α^5-di- *O*-(*β*-D-glucopyranosyl)-6-fluoropyridoxol **12** (5.1 mg, 7.5 mmol) with stably transfected MCF7-*lacZ* cells (5 x 10^6) in PBS (0.1 M, pH) 7.4, 600 *u*L) at 37 °C. Spectra were acquired in 51 s and enhanced with an exponential line broadening) 100 Hz. [DUFPOL) α^4, α^5- di-*O*-(*β*-D-glucopyranosyl)-6-fluoropyridoxol.]

Table 1 Acidities and ^{19}F NMR/pH Properties of DGFPOL, DUFPOL, DMFPOL, and FPOL in Saline at 25 °C[a]

pH indicators	DGFPOL	DUFPOL	DMFPOL	FPOL[24]
pK_a	7.95	8.08	8.18	8.20
δ_{Facid}	−8.34	−8.15	−7.44	−9.85
δ_{Fbase}	−19.05	−18.85	−18.15	−19.61

[a] Chemical shifts are given in parts per million (ppm) with respect to sodium trifluoroacetate.

^{19}F NMR chemical shift pH titration curve of DGFPOL, DUFPOL, and DMFPOL in 0.9% saline at 37 °C. [DGFPOL) α^4, α^5- di-*O*-(*β*-D-galactopyranosyl)-6-fluoropyridoxol; DUFPOL) α ^{4}, α^5-

di-*O*-(*β*-D-glucopyranosyl)-6-fluoropyridoxol; DMFPOL) α^4, α^5-di-*O*- (*β* -D-mannopyranosyl)-6-fluoropyridoxol.]

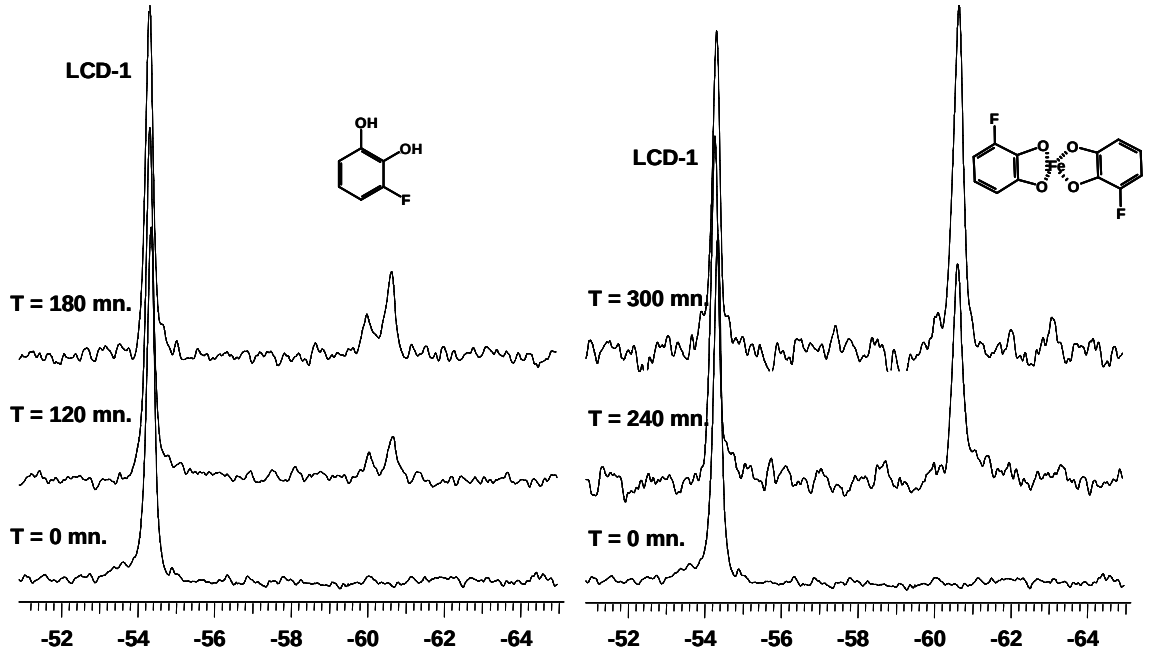

Figure 7 LCD-1 was stable in solution and gave a single sharp ^{19}F NMR signal. Addition of **LCD-1** to MCF7-*lacZ* cells caused rapid cleavage (8 μmol/min per million cells) generating two new signals (left spectra). When ferric ammonium citrate (FAC) was included, cells generated a purple solution indicative of Fe-complex formation (right spectra)**.** Now only a single resonance was observed, which we believe represents the trapped complex. We continue to explore the nature of these signals

Task 11

PFONPG and OFPNPG have been examined in vivo (Fig. 1). We are also exploring use of the "black stain" S-Gal™ as a potential proton MRI reporter for β-gal. Upon cleavage by beta-galactosidase in the presence of ferric ions (Fe^{3+}), the aglycone chelates iron to produce an intense black stain, which is not only visible, but also paramagnetic. For *in vivo* experiments

1.5x10^6 MCF7/WT or MCF7/LacZ tumor cells were implanted in both flanks of nude mice and allowed to grow to about 0.7 cm^3. Then 50 mg/kg S-gal-Na (3,4-cyclohexenoesculetin-β-*D*-galactopyranoside) sodium) and 25 mg/kg FAC (ferric ammonium citrate) in saline were injected intra tumorally. The whole abdomen was observed using a 2 cm volume coil at 4.7 T. Following intra tumor injection of S-gal + FAC into MCF7-LacZ tumor there was rapid development of intense contrast in the form of signal loss in T2 weighted images (Fig. 8)

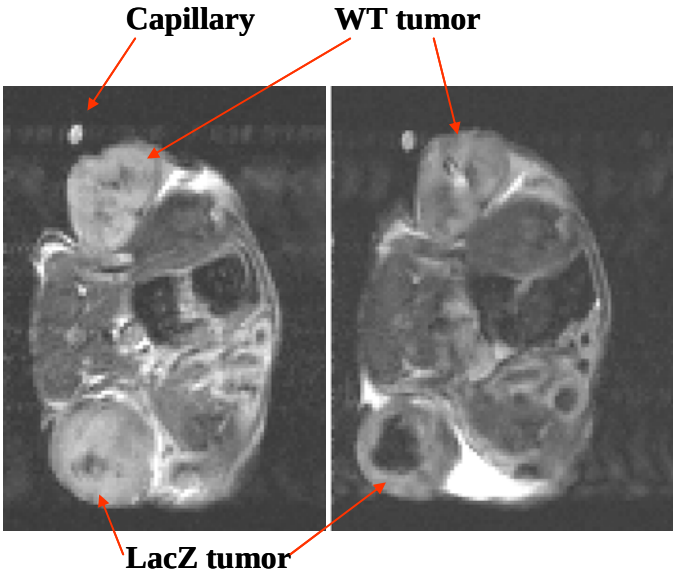

Figure 8. Mouse bearing MCF7 and MCF7/LacZ tumors was imaged using a 4.7T MR Scanner. The T2* weighted images were obtained before (left) and after (right) the intratumoral injection of S-gal-Na and FAC. (TR =500 ms, TE=15 ms, Flip angle=20, matrix=128x128)

Task 12 Ongoing We have published 7 manuscripts to date and are working on additional papers for imminent submission.

KEY RESEARCH ACCOMPLISHMENTS:

- We have generated breast tumor cell lines stably expressing high activity of β-galactosidase.

We have demonstrated ^{19}F NMR detection of β-gal activity in breast tumor cells *in vitro* and in tumors growing in mice. Meanwhile little activity was found in wild type cells and tumors.

- We have successfully synthesized a series of novel fluorine substituted phenylgalactosides as potential ^{19}F NMR reporter molecules for β-galactosidase activity.
- The fluorophenyl β-D-galactopyranosides are stable in saline, but are rapidly cleaved by the enzyme β-galactosidase.
- The fluorophenyl β-D-galactopyranosides provide a single ^{19}F NMR signal, which is invariant with pH. Enzyme cleavage produces a new signal well removed from the parent compound.
- Preliminary data show that the fluorophenyl β-D-galactopyranosides enter breast tumor cells. In wild type cells, the substrate is stable, but in cells transfected to express β-gal. There is cleavage releasing aglycone product as revealed by chemically shifted signal.
- Direct intra tumoral injection of substrates allows us to differentiate lacZ tumors versus wild type.
- Incorporation of trifluoromethyl groups enhances NMR signal to noise, though there is a smaller chemical shift response to cleavage.
- Prototype "smart" β-gal substrates have been synthesized using the pH reporter molecule 6-fluoropyridoxol (FPOL) in place of fluorophenol aglycones. FPOL is less toxic, but also less reactive with β-gal.
- Introduction of sugar moieties onto the FPOL skeleton enhances water solubility and reactivity with β-gal.

- A problem with the first generation substrates and products is that they are washed out of tumors. Based on our observations with S-gal we have synthesized a novel agent (LCD1), which forms a gel upon cleavage in the presence of Fe^{3+} ions and becomes trapped.
- We have found that S-gal provides a proton MRI contrast agent for detecting b-gal activity in tumors. The product is an insoluble paramagnetic precipitate.

REPORTABLE OUTCOMES:

New MCF7-LacZ stably transfected cell line.

New MCF7-LacZ-luc stably doubly transfected breast tumor cell line.

Published manuscripts

Year 1- one manuscript

Year 2- three manuscripts

Year 3

1. Yu J, Liu L, Kodibagkar VD, Cui W, **Mason RP**: Synthesis and Evaluation of Novel Enhanced Gene Reporter Molecules: Detection of β-Galactosidase Activity Using ^{19}F NMR of Trifluoromethylated Aryl β-*D*-Galactopyranosides", *Bioorg. Med. Chem.*, 2006,14, 326-33.
2. Yu JX, **Mason RP**: Synthesis and Characterization of Novel *lacZ* Gene Reporter Molecules: Detection of β-Galactosidase Activity Using ^{19}F NMR of Polyglycosylated Fluorinated Vitamin B_6, *J. Med. Chem*. 49:1991-9 (2006)
3. Kodibagkar VD, Yu J, Liu L, Hetherington H, **Mason RP** "Imaging B-galactosidase activity using ^{19}F CSI of lacZ gene-reporter molecule 2-fluoro-4-nitrophenol-B-D-galactopyranoside (OFPNPG)" *Magn. Reson. Imaging* in the press (2006)

Conference presentations

Year 1- 3 presentations

Year 2 - 6 presentations

Year 3

1. "Breast cancer gene therapy: development of novel non-invasive magnetic resonance assay to optimize efficacy", **R. P. Mason**, J. Yu., L. Liu, V. D. Kodibagkar, W Cui, and S. L. Brown, Era of Hope, P65-7, Philadelphia PA June (2005)
2. "Magnetic resonance assays of gene imaging constructs (MAGIC)", **R. P. Mason**, J. Yu, L. Liu, W. Cui V. Kodibagkar, Imaging in 2020, Jackson Hole, WY September (2005)
3. "A Novel Approach in the Development of ^{19}F NMR Reporter to Assess *LacZ* Gene Expression" J. X. Yu, Y. Ren, and **R. P. Mason**, Proc 14th ISMRM, 191, Seattle, May (2006)

CONCLUSIONS:

Gene therapy holds great promise for tracing breast cancer. A major current obstacle to implementation is assessment of gene expression in terms of heterogeneity and longevity in tissues. Reporter genes and associated molecules should allow assessment of gene expressions. To date successful reporters have been developed for nuclear imaging, but radionuclides can be difficult to handle and decay limiting shelf life detectability (6). Optical techniques are favored for gene assessment in small animals, but light penetration can limit utility (7). NMR facilitates assessment of deep tissues without radiation exposure. We have now demonstrated the feasibility of synthesizing an NMR reporter molecule to reveal activity of β-galactosidase, the primary tool

of molecular biologists to assess gene transfection. Significantly the molecules enter cells and are effective substrates. Moreover the ^{19}F NMR chemical shift unequivocally reveals enzyme activity. Differences in chemical shift associated with small molecular changes may further allow multiple substrates to be interrogated simultaneously allowing for two tumors to be interrogated simultaneously using unlocalized spectroscopy. Appropriate NMR pulse sequences allow distribution of substrate and product to be observed by imaging in phantoms. Second generation agents exhibit enhanced sensitivity to enzyme activity, accompanied by modified toxicity.

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7. Contag, C. H. and Ross, B. D. It's not just about anatomy: In vivo bioluminescence imaging as an eyepiece into biology. JMRI, *16:* 378-387, 2002.

APPENDICES:

1 Yu J, Liu L, Kodibagkar VD, Cui W, **Mason RP**: Synthesis and Evaluation of Novel Enhanced Gene Reporter Molecules: Detection of β-Galactosidase Activity Using ^{19}F NMR of Trifluoromethylated Aryl β-*D*-Galactopyranosides", *Bioorg. Med. Chem.*, 2006,14, 326-33.

2 Yu JX, **Mason RP**: Synthesis and Characterization of Novel *lacZ* Gene Reporter Molecules: Detection of β-Galactosidase Activity Using ^{19}F NMR of Polyglycosylated Fluorinated Vitamin B_6, *J. Med. Chem*. 49:1991-9 (2006)

3 Kodibagkar VD, Yu J, Liu L, Hetherington H, **Mason RP** "Imaging B-galactosidase activity using ^{19}F CSI of lacZ gene-reporter molecule 2-fluoro-4-nitrophenol-B-D-galactopyranoside (OFPNPG)" *Magn. Reson. Imaging* in the press (2006)

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Synthesis and evaluation of novel enhanced gene reporter molecules: Detection of β-galactosidase activity using ^{19}F NMR of trifluoromethylated aryl β-D-galactopyranosides

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Abstract—Gene therapy has emerged as a promising strategy for treatment of various diseases, but there is a pressing need for the development of non-invasive reporter techniques based on appropriate molecules and imaging modalities to assay gene expression. We now report the design, synthesis, and evaluation of novel enhanced reporter molecules, which reveal *lacZ* gene expression: trifluoromethylated aryl β-D-galactopyranosides. A series of five molecular structures were screened in solution and with stably transfected *lacZ* expressing human MCF7 breast cancer cells in vitro. *p*-Trifluoromethyl-*o*-nitrophenyl β-D-galactopyranoside (**PCF_3ONPG**) was found to exhibit valuable properties including a single ^{19}F NMR signal, stability in aqueous solution and with wild type cells, but a chemical shift response to enzyme cleavage ($\Delta\delta$ = 1.14 ppm) in breast cancer cells transfected to stably express *lacZ*.

1. Introduction

Strategies for identifying exogenous gene activity have been presented using radionuclide imaging,[1,2] optical imaging,[3,4] and NMR.[5,6] In some cases, natural substrates are used, such as detection of fluorescent molecules or bioluminescence, but in other cases exogenous substrates have been designed to probe enzyme (viz., gene) activity. Recently, attention has turned to β-galactosidase (β-gal), since its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction. Diverse colorimetric substrates have been developed suitable for histology.[7,8] Tung et al.[9] reported a near infrared active substrate and Louie et al.[10] presented a proton MRI contrast agent. We have demonstrated the feasibility of using ^{19}F NMR to detect chemical shift changes accompanying enzyme-induced cleavage of fluorogalactopyranosides.[11–14] We now report the synthesis of a series of trifluoromethyl (CF_3) aryl β-D-galactopyranosides designed to provide enhanced signal. We report synthesis, relevant characteristics as β-gal substrates, and evaluation of their use to detect *lacZ* gene expression in breast cancer cells. Relative merits are compared with those of previous substrates.

2. Designs and synthesis

β-Galactosidase (β-gal) catalyzes the hydrolysis of galactopyranosides by cleavage of the C–O bonds between D-galactose and the aglycone.[15] However, the enzyme shows remarkably broad substrate specificity. Based on our previous studies using a single fluorine atom as ^{19}F NMR sensitive reporter of β-gal activity,[11–14] it appeared that introduction of a CF_3 group could be advantageous. Inherent signal to noise would be improved, allowing lower concentrations of reporter molecule to be applied, and hence, reducing issues of toxicity or substrate solubility. CF_3– groups are widely used in pharmaceuticals and agrochemicals since they resist enzyme degradation and the typical toxicity of mono- and difluoromethyl groups.[14] Moreover, it has been observed that hydrogen bonding between the active site of the enzyme and the hydroxyl groups of the glycosidic substrate is important in the formation of the enzyme–substrate complex.[16–18] Introduction of strong electron-withdrawing CF_3– group could increase

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the tendency to form the enzyme–substrate complex through the action of the fluorine as an acceptor in hydrogen bonding interactions in the 'glycosylation' step and make the phenolate anion a better leaving group in the 'deglycosylation' step.[19]

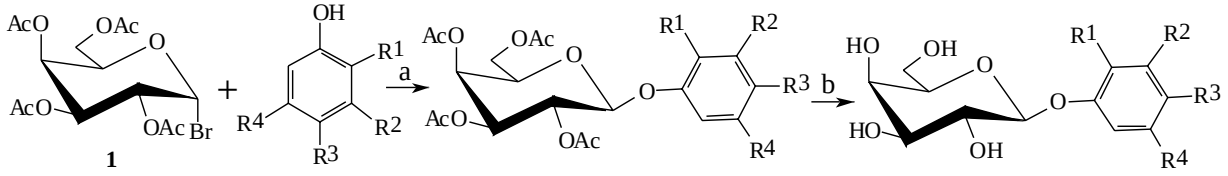
Following the successful high yield phase-transfer catalysis approach to the stereoselective syntheses of fluorinated aryl β-D-galactopyranosides,[12,13] this versatile synthetic method was chosen for preparation of the target compounds **9**–**18** starting with commercially available trifluoromethylphenolic aglycones **2**–**8** (Fig. 1). The aglycones **2**–**8** reacted at 50 °C with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**1**) in a dichloromethane-aqueous biphasic system (pH 8–9) using tetrabutylammonium bromide (TBAB) as the phase-transfer catalyst, affording trifluoromethyl aryl β-D-galactopyranoside tetraacetates **9**–**12** in near quantitative yield. However, **13** was obtained in poor yield (20%) and two of the trifluoromethyl phenols (3-trifluoromethylphenol **7** and 4-trifluoromethylphenol **8**) proved to be unreactive. To our knowledge molecules **9**–**18** are new, though we note that an isomer 4-nitro-2-trifluoromethylphenyl β-D-galactopyranoside has been reported previously in a patent related to CEDIA (cloned enzyme donor immunoassay).[20] That work did not appear to exploit the ^{19}F NMR properties, but rather colorimetric changes accompanying β-gal induced cleavage. They reported a very poor yield using an alternate synthetic approach, though it may be characteristic of *o*-CF_3 groups since this was least successful in our hands.

The anomeric β-D-configuration of compounds **9**–**13** in the $^4C_1$ chair conformation was unambiguously established on the basis of the observed ^{1}H NMR chemical shifts (δ_H 4.98–5.25 ppm) of the anomeric protons, and the $J_{1,2}$ (J ~8 Hz) and $J_{2,3}$ (J ~10 Hz) coupling constants. The signals of the ^{13}C NMR spectra of **9**–**13** were assigned by comparison with the chemical shifts of *p*-nitrophenyl β-D-galactopyranosides.[13,21] As expected, the anomeric carbon resonances appeared at 98–101 ppm in accord with the β-D-configuration.

Deacetylation of **9**–**13** with NH_3/MeOH from 0 °C to room temperature gave the free galactopyranosides **14**–**18** in quantitative yield. The signals of the ^{1}H NMR spectra of **14**–**18** were assigned by ^{1}H–^{1}H COSY spectra and D_2O exchange. The ^{1}H NMR chemical shifts (δ_H 5.00–5.15 ppm) of the anomeric protons and the $J_{1,2}$ (J ~8 Hz) and $J_{2,3}$ (J 9–11 Hz) coupling constants showed that the free galactopyranosides **14**–**18** retained the anomeric β-D-configuration with the $^4C_1$ chair conformation.

3. ^{19}F NMR

^{19}F NMR spectra of the trifluoromethylphenyl β-D-galactopyranosides **14**–**18** were recorded in aqueous solutions with sodium trifluoroacetate (NaTFA) as an external chemical shift standard. Compounds **14**–**18** each gave a single narrow ^{19}F NMR signal between δ 12–16 ppm essentially invariant ($\Delta\delta \leqslant 0.02$ ppm) with pH in the range 3 to 12 and temperatures from 25 to 37 °C in whole rabbit blood, 0.9% saline, or PBS. Addition of β-gal (G-2513) to **14**–**17** in PBS buffer (0.1 M, pH 7.4) at 37 °C led to rapid hydrolysis releasing the aglycones **2**–**5**, which appeared as single narrow ^{19}F signals shifted downfield (Table 1). Compound **18** was cleaved comparatively slowly. The relative efficacy of **14** (**PCF_3ONPG**) and that of our previously reported *o*-fluoro-*p*-nitrophenyl β-D-galactopyranoside (**OFPNPG**) as β-gal substrate are shown in Figure 2. As expected, **14** provides about 3 times more signal, while cleavage rates are similar. Comparison of β-gal hydrolytic kinetics of **14**–**18** (Fig. 3) showed that each proceeded



Compounds	R^1	R^2	R^3	R^4
2, 9, 14	**NO_2**	**H**	**CF_3**	**H**
3, 10, 15	**H**	**CF_3**	**NO_2**	**H**
4, 11, 16	**Cl**	**CF_3**	**H**	**H**
5, 12, 17	**Cl**	**H**	**H**	**CF_3**
6, 13, 18	**CF_3**	**H**	**H**	**H**
7	**H**	**CF_3**	**H**	**H**
8	**H**	**H**	**CF_3**	**H**

Figure 1. The reactions and the structures of **1**–**18**. Reaction and condition: (a) CH_2Cl_2–H_2O, pH 8–9, 50 °C, TBAB, ~1 h, near quantitative yield except **13** in only 20% yield; (b) NH_3–MeOH, 0 °C → rt, 24 h, quantitative yields.

Table 1. ^{19}F chemical shifts[a] and hydrolytic rates by β-gal[b]

	14	15	16	17	18
$\delta_{F(substrate)}$	13.40	15.23	13.24	12.70	14.11
$\delta_{F(product)}$	14.54	15.43	13.94	12.95	14.61
$\Delta\delta_F$	1.14	0.20	0.70	0.25	0.50
$v_{(\mu mol/min/U)}$	33.0	52.8	39.6	46.2	2.61

[a] ppm with respect to aq NaTFA.
[b] β-Gal (G-2513, 11 U) at 37 °C in PBS buffer (0.1 M, pH 7.4).

monotonically indicating straightforward first-order kinetics for all substrates and that the liberated aglycones **2**–**6** did not inhibit the β-gal. The substrates **14**–**17** exhibited rates in excess of 33 μmol/min/U exceeding those of *p*-fluoro-*o*-nitrophenyl β-D-galactopyranoside (**PFONPG**; 19 μmol/min/U), **OFPNPG** (32 μmol/min/U), and even *o*-nitrophenyl β-D-galactopyranoside (**ONPG**; 32 μmol/min/U), which we have reported previously.[11,13] The low hydrolysis rate of **18** may be due to the formation of an intramolecular F···H hydrogen bond between the 2-CF_3 and C_1–H or steric effects, which plays an important role in the hydrolytic process rate.[19]

As expected, trifluoromethylphenyl β-D-galactopyranosides showed enhanced ^{19}F signal intensity on a molar basis compared with analogous fluorophenyl β-D-galactopyranosides, but the ^{19}F chemical shift changes were much smaller (Fig. 2, Table 1). The chemical shift ($\Delta\delta$ 1.14 ppm) accompanying cleavage of **14** is sufficient for investigations in vivo, but the other substrates **15**–**18** gave smaller values, which may be insufficient for effective studies. The aglycone *p*-trifluoromethyl-*o*-nitrophenol (**2**, **PCF_3ONP**) also exhibits a ^{19}F NMR chemical shift in response to pH ($\Delta\delta$ = ~1.00 ppm) in the range of pH 4–7 (Fig. 4). Henderson–Hasselbalch coefficients are pK_a = 5.6, δ_{acid} = 13.49 ppm, δ_{base} = 14.52, but importantly there is no overlap with the chemical shift of the substrate **14**.

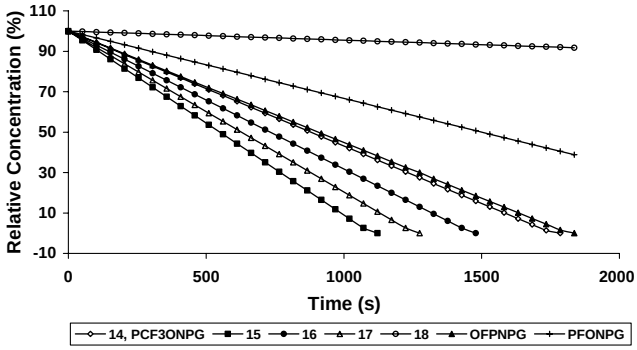


Figure 3. Relative hydrolysis time courses of **14**–**18** (6.0 mmol), **OFPNPG**, and **PFONPG** (5.4 mmol) by β-gal (6 U) in PBS (0.1 M, 600 μL) at 37 °C.

4. In vitro evaluation

CF_3– groups are often associated with increased lipophilicity. As expected, **PCF_3ONPG** has comparatively lower aqueous solubility than either **OFPNPG** or **PFONPG**. However, the higher ^{19}F signal intensity and sensitivity to β-gal allow use of lower concentrations of **PCF_3ONPG**, potentially circumventing issues of toxicity. **PCF_3ONPG** was stable in aqueous solution in the pH range 3–12 at temperatures from 25 to 37 °C over 5 days. Toxicity was evaluated for both aglycone **PCF_3ONP** and conjugate **PCF_3ONPG** using both wild type and *lacZ* expressing human MCF7 breast cancer

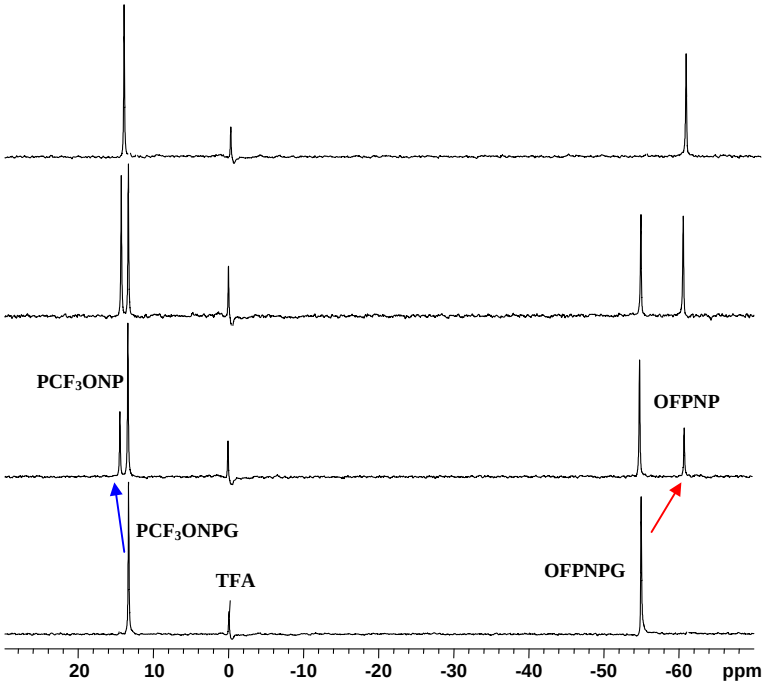


Figure 2. ^{19}F NMR spectra of **PCF_3ONPG** (1.1 mg, 3 mmol) and **OFPNPG** (2.87 mg, 9 mmol) with 1:3 molar ratios in the simultaneous hydrolysis by β-gal (11 U) in PBS (0.1 M, pH 7.4, 600 μL) at 37 °C.

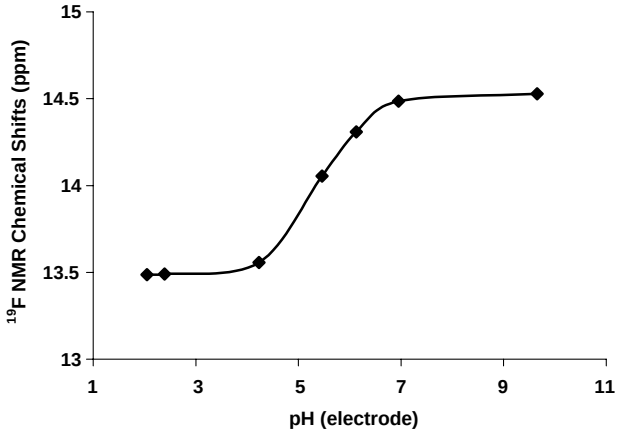


Figure 4. ^{19}F NMR chemical shift pH titration curve of **2** (**PCF_3ONP**) in saline at 37 °C.

cells. Cell viability assays[22] showed that the aglycone **PCF_3ONP** exhibited significant cytotoxicity even at 100 μM with both cell clones (Fig. 5). No toxicity was observed up to 1 mM for **PCF_3ONPG** over 96 h for wild type cells, but some toxicity was found with the *lacZ* expressing cells, presumably due to liberation of the aglycone.

When **PCF_3ONPG** was incubated with MCF7-WT cells for 5 h in PBS buffer at 37 °C under 5% CO_2 in air with 95% humidity, no changes were observed in the ^{19}F NMR spectra. However, addition of **PCF_3ONPG** to cells stably transfected to express β-gal led to cleavage in a smooth monotonic manner releasing the aglycone **PCF_3ONP** (40.0 μmol/min per million MCF7-*lacZ* cells, Figs. 6, 7).

While the chemical shift response of the trifluoromethyl reporters is modest, we demonstrate that it is sufficient for chemical shift selective imaging (CSI) and we have observed the effect of MCF7-*lacZ* cells on **PCF_3ONPG** in vitro (Fig. 8).

5. Conclusion

The phase-transfer approach to synthesizing phenyl galactosides developed previously[13] was also appropriate for several of the trifluoromethyl galactosides. The substrates are stable in aqueous solution and with wild type cancer cells, but the CF_3 agents are responsive to β-gal activity with rates exceeding those of the fluorophenyl analogs. Signal to noise is enhanced and although the ^{19}F NMR chemical shift response to enzyme cleavage is smaller, it is adequate for detecting hydrolysis with **PCF_3ONPG**. Overall, the trifluoromethyl galactosides show promise as reporter molecules for β-gal activity and we are initiating investigations of *lacZ* expressing tumors in animals.

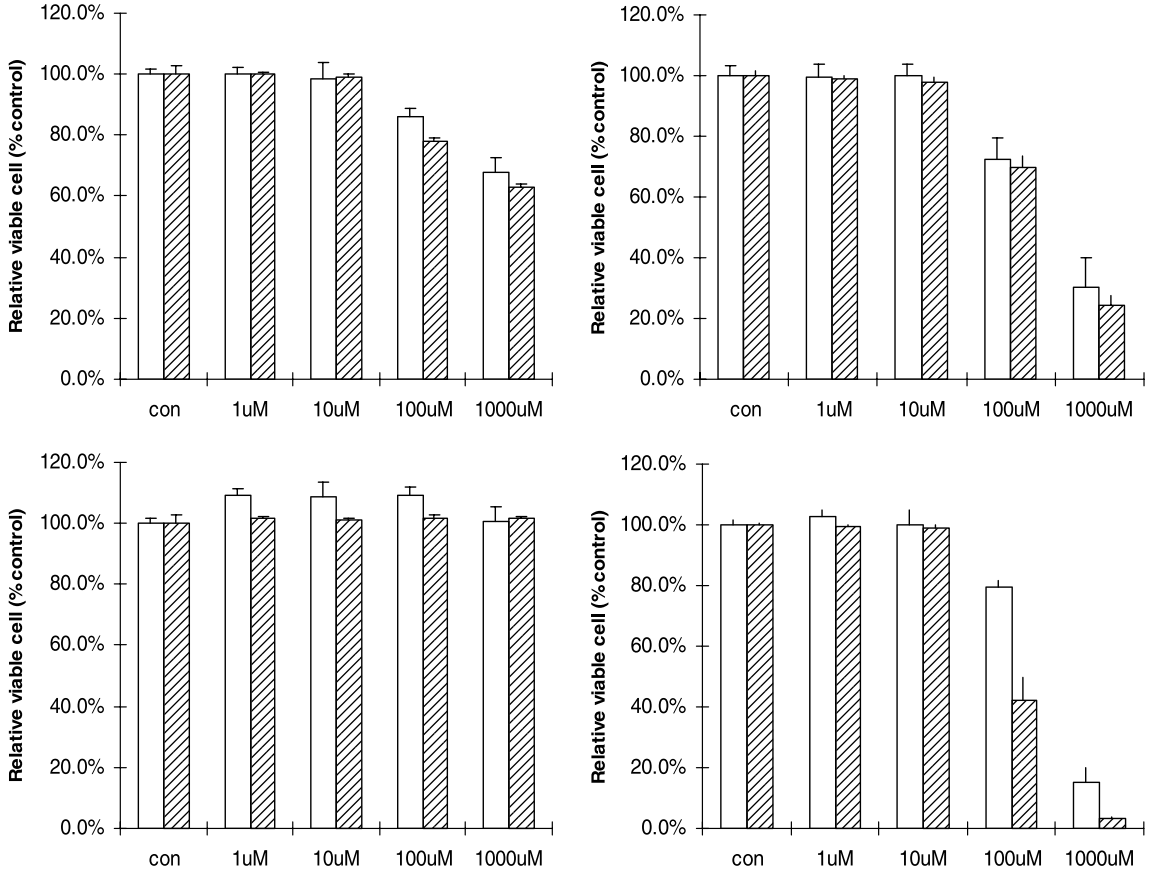


Figure 5. Cell viability of human MCF7 breast cancer cells in PBS (pH 7.4) with respect to exposure to substrate **14** (left panels) or aglycone **2** (right panels). Upper panels MCF7-*lacZ* cells; lower panels MCF7-WT. Open bars 48 h exposure; hatched bars 96 h exposure.

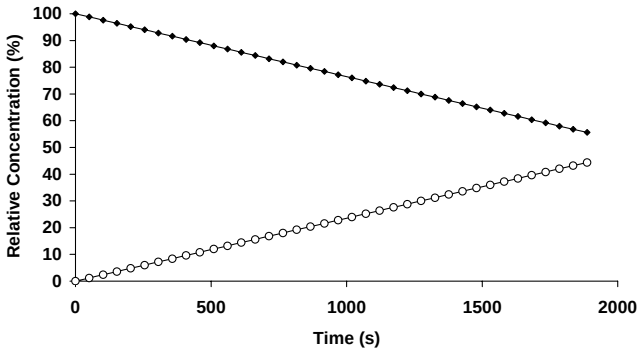


Figure 6. Hydrolysis of **14** (♦, 5.0 mmol) to **2** (○) by stably transfected MCF7-*lacZ* breast cancer cells (1.75×10^6) in PBS buffer at 37 °C.

6. Experimental

6.1. General methods

NMR spectra were recorded on a Varian Inova 400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C, 376 MHz for ^{19}F) with $CDCl_3$ or DMSO-d_6 as solvents. ^{1}H and ^{13}C chemical shifts are referenced to TMS as internal standard and ^{19}F to dil sodium trifluoroacetate (NaTFA) in a capillary as external standard. All compounds were characterized by acquisition of ^{1}H, ^{13}C, DEPT, ^{1}H–^{1}H, and COSY experiments at 25 °C and ^{19}F spectra at 37 °C. Imaging experiments used a Varian INOVA Unity scanner 4.7 T (188.2 MHz) with a 2D spin echo CSI sequence (FOV = 30 × 30 mm, spectral window = 30 ppm, slice thickness: 10 mm, matrix = 16 × 16, and TR/TE = 1000/12 ms in 4.5 min per image). Microanalyses were performed on a Perkin-Elmer 2400CHN microanalyzer. Solutions in organic solvents were dried with anhydrous sodium sulfate and concentrated in vacuo below 45 °C. Column chromatography was performed on silica gel (200–300 mesh) by elution with cyclohexane–EtOAc and silica gel GF_{254} (Aldrich Chemical Company, St. Louis, MO) was used for analytical TLC. Detection was effected by spraying the plates with 5% ethanolic H_2SO_4 (followed by heating at 110 °C for 10 min) or by direct UV illumination of the plate.

For enzyme kinetic experiments, **PCF_3ONPG** (2.2 mg, 6 mmol) was dissolved in PBS (0.1 M, pH 7.4, 573 μL) and a PBS solution of β-gal (27 μL, G-2513 from *Escherichia coli*, 0.22 U/μL, Aldrich) was added and NMR data were acquired immediately at 37 °C.

Human MCF7 breast cancer cells were stably transfected with recombinant vector phCMV/*lacZ*, which inserted the *E. coli lacZ* gene (from pSV-β-gal vector, Promega) to high expression human cytomegalovirus (CMV) immediate-early enhancer/promoter vector phCMV (Gene Therapy Systems, Inc) using GenePORTER2 (Gene Therapy Systems, Inc). For MCF7 cells, clonal selection was applied to identify those cells with highest β-gal expression. Control (wild type) and transfected (*lacZ*) cells were grown in culture dishes under standard conditions and harvested. **PCF_3ONPG** (2.2 mg) in PBS (70 μL) was added to a suspension of 10^6 cells in PBS (530 μL) and ^{19}F NMR spectra were acquired immediately and again after incubation for various times up to 5 h at 37 °C.

The sensitivities of MCF7-WT and -*lacZ* cells to **PCF_3ONPG** and **PCF_3ONP** were quantified using the Crystal Violet Mitogenic Assay[23] performed in triplicate using 24-well plates seeded with 2×10^4 cells per well in 1 mL DMEM supplemented with 10% FBS and 2 mM

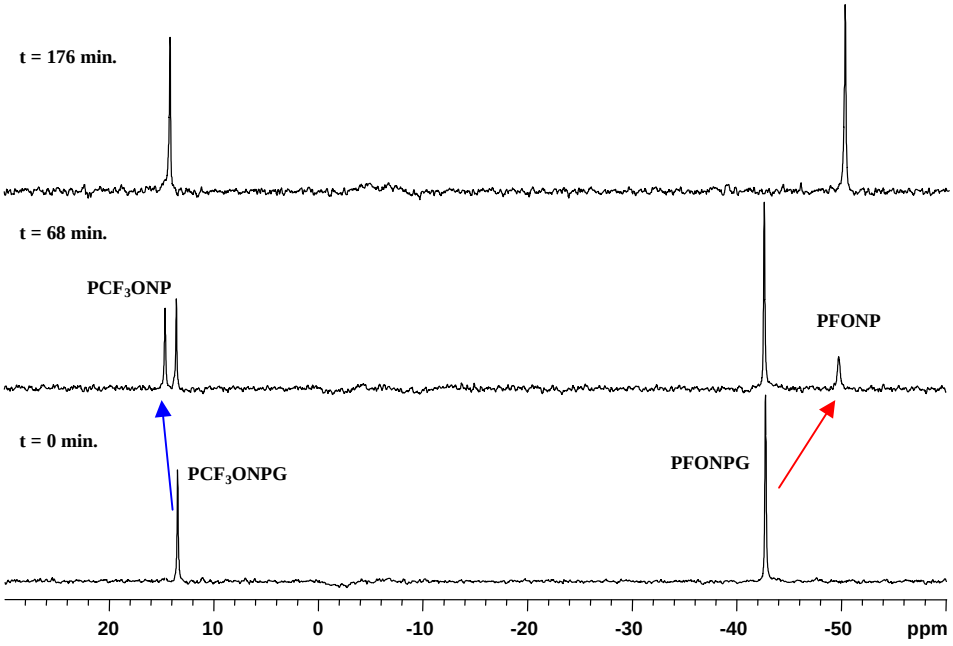


Figure 7. ^{19}F NMR spectra of **PCF_3ONPG** (1.7 mg, 4.5 mmol) and **PFONPG** (6.0 mg, 18.8 mmol) showing simultaneous hydrolysis by stably transfected MCF7-*lacZ* cells (1.75×10^6) in PBS (0.1 M, pH 7.4, 600 μL) at 37 °C. Each spectrum acquired in 51 s.

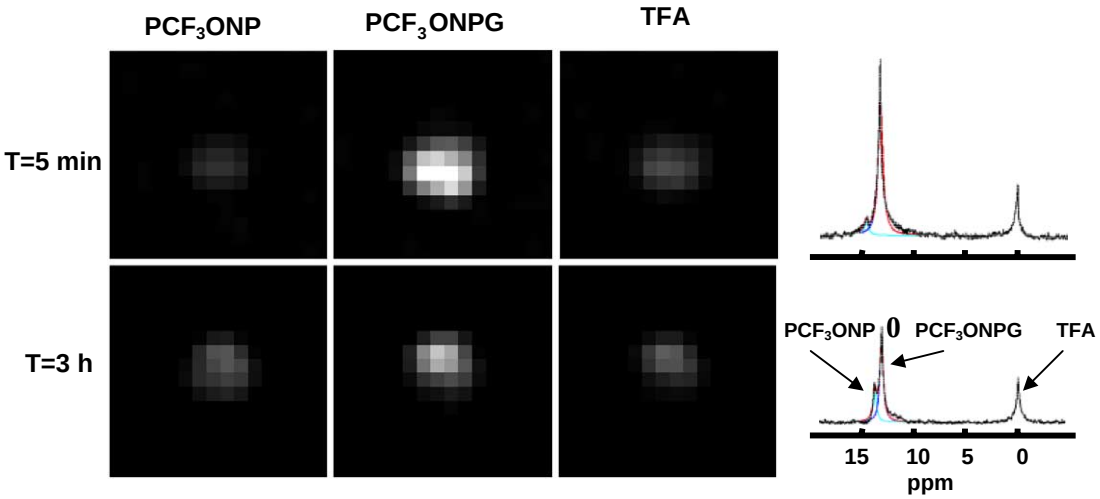


Figure 8. ^{19}F CSI of **PCF$_3$ONPG** (13.2 mg) and **TFA** (2.0 mg) during hydrolysis by stably transfected MCF7-*lacZ* cells (10^7) in PBS (0.1 M, pH 7.4, 700 μL) at 20 °C. Upper images show data acquired in 4.5 min, ten minutes after addition of substrate to cells. The lower images were acquired 3 h later. At right are corresponding ^{19}F NMR spectra obtained from a single voxel in the image. The curve fits are also presented to demonstrate the deconvolution to allow CSI.

glutamine. After 24 h incubation, the medium was replaced with fresh DMEM containing 0.1% DMSO and various concentrations of **PCF$_3$ONPG** or **PCF$_3$ONP** (0–1 mM) and incubated for 48 or 96 h, followed by the Crystal Violet Mitogenic Assay.

6.2. Trifluoromethylphenyl β-D-galactopyranoside tetraacetates 9–13

6.2.1. General procedure. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (Sigma) (**1**) (1 mmol) and tetrabutyl-ammonium bromide (0.48 g, 1.5 mmol) in CH_2Cl_2 (5 mL) was stirred vigorously at 50 °C with the solution of fluorophenols (**2**–**8**) (1.2 mmol) in H_2O (5 mL; pH 8–9) until TLC showed complete reaction (~1 h). The organic layer was separated, washed, dried (Na_2SO_4), and evaporated under reduced pressure to give a syrup, which was purified by column chromatography on silica gel to give trifluoromethylphenyl β-D-galactopyranoside tetraacetates **9**–**13**.

2-Nitro-4-trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **9** (0.54 g, 99%) as white crystals, R_f 0.38 (3:2 cyclohexane/EtOAc), δ_H: 8.07 (1H, d, J = 2.0 Hz, Ar-H), 7.79 (1H, dd, J = 1.6, 7.2 Hz, Ar-H), 7.48 (1H, d, J = 8.8 Hz, Ar-H), 5.17 (1H, d, $J_{1,2}$ = 7.6 Hz, H-1), 5.57 (1H, dd, $J_{2,3}$ = 10.4 Hz, H-2), 5.12 (1H, dd, $J_{3,4}$ = 3.2 Hz, H-3), 5.48 (1H, d, $J_{4,5}$ = 3.2 Hz, H-4), 4.13 (1H, m, H-5), 4.24 (1H, dd, $J_{5,6a}$ = 4.4 Hz, $J_{6a,6b}$ = 11.2 Hz, H-6a), 4.18 (1H, dd, $J_{5,6b}$ = 5.6 Hz, H-6b), 2.19, 2.15, 2.10, 2.01 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.49, 170.31, 169.45 (4× $CH_3\mathbf{C}O$), 140.93 (Ar-$C_{1'}$), 151.87 (Ar-$C_{2'}$), 130.74 (q, $^3J_{F-C}$ = 3.8 Hz, Ar-$C_{3'}$), 126.14 (q, $^2J_{F-C}$ = 34.0 Hz, Ar-$C_{4'}$), 123.10 (q, $^3J_{F-C}$ = 3.9 Hz, Ar-$C_{5'}$), 119.59 (Ar-$C_{6'}$), 122.82 (q, $^1J_{F-C}$ = 284.0 Hz, CF_3), 100.46 (C-1), 67.80 (C-2), 70.55 (C-3), 66.79 (C-4), 71.89 (C-5), 61.55 (C-6), 20.84, 20.80, 20.78, 20.73 (4× $\mathbf{C}H_3CO$) ppm. Anal. Calcd for $C_{21}H_{22}NO_{12}F_3$ (%): C, 46.92; H, 4.13; N, 2.61. Found: C, 46.90; H, 4.10; N, 2.58.

4-Nitro-3-trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **10** (0.54 g, 99%) as syrup, R_f 0.36 (3:2 cyclohexane/EtOAc), δ_H: 7.98 (1H, d, J = 8.8 Hz, Ar-H), 7.44 (1H, s, Ar-H), 7.28 (1H, d, J = 9.2 Hz, Ar-H), 5.25 (1H, d, $J_{1,2}$ = 8.0 Hz, H-1), 5.57 (1H, dd, $J_{2,3}$ = 10 Hz, H-2), 5.17 (1H, dd, $J_{3,4}$ = 2.8 Hz, H-3), 5.50 (1H, d, $J_{4,5}$ = 3.2 Hz, H-4), 4.15 (1H, m, H-5), 4.18 (2H, m, H-6), 2.20, 2.09, 2.07, 2.03 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.61, 170.26, 170.16, 169.45 (4× $CH_3\mathbf{C}O$), 142.91 (Ar-$C_{1'}$), 116.15 (q, $^3J_{F-C}$ = 6.1 Hz, Ar-$C_{2'}$), 126.18 (q, $^2J_{F-C}$ = 34.4 Hz, Ar-$C_{3'}$), 120.07 (Ar-$C_{4'}$), 127.91 (Ar-$C_{5'}$), 159.31 (Ar-$C_{6'}$), 121.76 (q, $^1J_{F-C}$ = 271.6 Hz, CF_3), 98.70 (C-1), 68.30 (C-2), 70.65 (C-3), 67.01 (C-4), 71.99 (C-5), 61.95 (C-6), 20.82, 20.79, 20.71, 20.67 (4× $\mathbf{C}H_3CO$) ppm. Anal. Calcd for $C_{21}H_{22}NO_{12}F_3$ (%): C, 46.92; H, 4.13; N, 2.61. Found: C, 46.89; H, 4.11; N, 2.60.

2-Chloro-3-trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **11** (0.50 g, 95%) as syrup, R_f 0.61 (3:2 cyclohexane/EtOAc), δ_H: 7.46 (1H, dd, J = 1.2, 6.6 Hz, Ar-H), 7.40 (1H, d, J = 8.4 Hz, Ar-H), 7.32 (1H, dd, J = 7.8, 8.4 Hz, Ar-H), 4.98 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 5.59 (1H, dd, $J_{2,3}$ = 10.5 Hz, H-2), 5.11 (1H, dd, $J_{3,4}$ = 3.6 Hz, H-3), 5.48 (1H, d, $J_{4,5}$ = 3.6 Hz, H-4), 4.06 (1H, m, H-5), 4.25 (1H, dd, $J_{5,6a}$ = 6.6 Hz, $J_{6a,6b}$ = 11.7 Hz, H-6a), 4.16 (1H, dd, $J_{5,6b}$ = 6.0 Hz, H-6b), 2.20, 2.10, 2.06, 2.02 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.45, 170.42, 170.34, 169.42 (4× $CH_3\mathbf{C}O$), 153.82 (Ar-$C_{1'}$), 122.39 (q, $^3J_{F-C}$ = 3.6 Hz, Ar-$C_{2'}$), 130.11 (q, $^2J_{F-C}$ = 20.6 Hz, Ar-$C_{3'}$), 122.39 (q, $^3J_{F-C}$ = 3.4 Hz, Ar-$C_{4'}$), 127.42 (Ar-$C_{5'}$), 132.58 (Ar-$C_{6'}$), 124.01 (q, $^1J_{F-C}$ = 282.5 Hz, CF_3), 100.83 (C-1), 68.28 (C-2), 70.69 (C-3), 66.87 (C-4),

71.47 (C-5), 61.42 (C-6), 20.92, 20.77, 20.70 (4× **C**H_3CO) ppm. Anal. Calcd for $C_{21}H_{22}O_{10}ClF_3$ (%): C, 47.90; H, 4.22. Found: C, 47.89; H, 4.20.

2-Chloro-5-trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **12** (0.51 g, 96%) as white crystals, R_f 0.56 (3:2 cyclohexane/EtOAc), δ_H: 7.51 (1H, s, Ar-H), 7.49 (1H, dd, J = 1.8, 3.6 Hz, Ar-H), 7.30 (1H, dd, J = 1.2, 7.8 Hz, Ar-H), 5.02 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 5.60 (1H, dd, $J_{2,3}$ = 10.2 Hz, H-2), 5.12 (1H, dd, $J_{3,4}$ = 3.6 Hz, H-3), 5.48 (1H, d, $J_{4,5}$ = 3.6 Hz, H-4), 4.12 (1H, m, H-5), 4.22 (1H, dd, $J_{5,6a}$ = 4.2 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6a), 4.17 (1H, dd, $J_{5,6b}$ = 7.8 Hz, H-6b), 2.20, 2.10, 2.07, 2.02 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.70, 170.27, 170.17, 169.40 (4× CH_3**C**O), 152.97 (Ar-$C_{1'}$), 109.92 (Ar-$C_{2'}$), 131.03 (Ar-$C_{3'}$), 115.13 (q, $^3J_{F-C}$ = 3.0 Hz, Ar-$C_{4'}$), 130.34 (q, $^2J_{F-C}$ = 22.2 Hz, Ar-$C_{5'}$), 121.06 (q, $^3J_{F-C}$ = 2.9 Hz, Ar-$C_{6'}$), 122.59 (q, $^1J_{F-C}$ = 282.0 Hz, CF_3), 100.65 (C-1), 68.13 (C-2), 70.62 (C-3), 67.21 (C-4), 71.95 (C-5), 62.31 (C-6), 20.89, 20.73, 20.50 (4× **C**H_3CO) ppm. Anal. Calcd for $C_{21}H_{22}O_{10}ClF_3$ (%): C, 47.90; H, 4.22. Found: C, 47.88; H, 4.21.

2-Trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside **13** (0.30 g, 20%) as white crystals, R_f 0.54 (3:2 cyclohexane/EtOAc), δ_H: 7.60 (1H, dd, J = 1.2, 7.8 Hz, Ar-H), 7.50 (1H, m, Ar-H), 7.27 (1H, dd, J = 1.8, 6.6 Hz, Ar-H), 7.16 (1H, dd, J = 7.8 Hz, Ar-H), 5.06 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 5.58 (1H, dd, $J_{2,3}$ = 10.5 Hz, H-2), 5.12 (1H, dd, $J_{3,4}$ = 3.6 Hz, H-3), 5.47 (1H, dd, $J_{4,5}$ = 0.6 Hz, H-4), 4.11 (1H, m, H-5), 4.27 (1H, dd, $J_{5,6a}$ = 6.0 Hz, $J_{6a,6b}$ = 11.4 Hz, H-6a), 4.17 (1H, dd, $J_{5,6b}$ = 6.0 Hz, H-6b), 2.20, 2.08, 2.05, 2.01 (12H, 4s, 4× CH_3CO) ppm; δ_C: 170.26, 170.20, 170.05, 169.10 (4× CH_3**C**O), 154.59 (Ar-$C_{1'}$), 120.10 (q, $^2J_{F-C}$ = 21.0 Hz, Ar-$C_{2'}$), 127.03 (q, $^3J_{F-C}$ = 3.4 Hz, Ar-$C_{3'}$), 122.80 (Ar-$C_{4'}$), 116.69 (Ar-$C_{5'}$), 133.30 (Ar-$C_{6'}$), 129.78 (q, $^1J_{F-C}$ = 208.4 Hz, CF_3), 99.79 (C-1), 67.85 (C-2), 70.73 (C-3), 66.76 (C-4), 71.20 (C-5), 61.40 (C-6), 20.59, 20.36 (4× **C**H_3CO) ppm. Anal. Calcd for $C_{21}H_{23}O_{10}F_3$ (%): C, 51.21; H, 4.71. Found: C, 51.19; H, 4.69.

6.3. Trifluoromethylphenyl β-D-galactopyranosides 14–18

6.3.1. General procedure. A solution of trifluoromethylphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**9**–**13**) (0.4 g) in anhydrous MeOH (15 mL) containing 0.5 M NH_3 was vigorously stirred from 0 °C to room temperature overnight until TLC showed complete reaction and evaporated to dryness in vacuo. Chromatography of the crude syrup on silica gel with EtOAc–MeOH afforded the free β-D-galactopyranosides **14**–**18** in quantitative yield.

2-Nitro-4-trifluoromethylphenyl β-D-galactopyranoside **14** as white crystals, R_f 0.35 (1:9 MeOH/EtOAc), δ_H: 8.28 (1H, d, J = 2.0 Hz, Ar-H), 7.99 (1H, dd, J = 2.0, 9.2 Hz, Ar-H), 7.59 (1H, d, J = 8.8 Hz, Ar-H), 5.15 (1H, d, $J_{1,2}$ = 7.6 Hz, H-1), 3.55 (1H, dd, $J_{2,3}$ = 8.9 Hz, H-2), 3.50 (1H, dd, $J_{3,4}$ = 6.0 Hz, H-3), 3.47 (1H, d, $J_{4,5}$ = 6.0 Hz, H-4), 3.42 (1H, m, H-5), 3.67 (1H, m, H-6), 5.24 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 4.8 Hz, HO-2), 4.63 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 4.4 Hz, HO-3), 4.92 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 6.0 Hz, HO-4), 4.69 (1H, t, $J_{H\text{-}6,OH\text{-}6}$ = 5.4, 5.6 Hz, HO-6) ppm; δ_C: 152.18 (Ar-$C_{1'}$), 139.97 (Ar-$C_{2'}$), 130.82 (q, $^3J_{F-C}$ = 3.9 Hz, Ar-$C_{3'}$), 122.06 (q, $^2J_{F-C}$ = 33.8 Hz, Ar-$C_{4'}$), 122.42 (q, $^3J_{F-C}$ = 3.8 Hz, Ar-$C_{5'}$), 117.91 (Ar-$C_{6'}$), 123.33 (q, $^1J_{F-C}$ = 270.9 Hz, CF_3), 100.95 (C-1), 69.97 (C-2), 73.29 (C-3), 68.03 (C-4), 76.04 (C-5), 60.30 (C-6) ppm. Anal. Calcd for $C_{13}H_{14}NO_8F_3$ (%): C, 42.27; H, 3.82; N, 3.79. Found: C, 42.25; H, 3.80; N, 3.77.

4-Nitro-3-trifluoromethylphenyl β-D-galactopyranoside **15** as white crystals, R_f 0.40 (1:9 MeOH/EtOAc), δ_H: 8.16 (1H, d, J = 8.8 Hz, Ar-H), 7.53 (1H, d, J = 2.8 Hz, Ar-H), 7.49 (1H, dd, J = 2.8, 8.8 Hz, Ar-H), 5.08 (1H, d, $J_{1,2}$ = 7.6 Hz, H-1), 3.53 (1H, dd, $J_{2,3}$ = 10.0 Hz, H-2), 3.49 (1H, dd, $J_{3,4}$ = 5.2 Hz, H-3), 3.44 (1H, d, $J_{4,5}$ = 2.4 Hz, H-4), 3.61 (1H, m, H-5), 3.68 (2H, m, H-6), 5.33 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 4.8 Hz, HO-2), 4.60 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 4.8 Hz, HO-3), 4.96 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 5.6 Hz, HO-4), 4.70 (1H, dd, $J_{H\text{-}6,OH\text{-}6}$ = 5.2, 5.4 Hz, HO-6) ppm; δ_C: 160.45 (Ar-$C_{1'}$), 115.95 (q, $^3J_{F-C}$ = 6.1 Hz, Ar-$C_{2'}$), 123.67 (q, $^2J_{F-C}$ = 32.8 Hz, Ar-$C_{3'}$), 141.10 (Ar-$C_{4'}$), 120.32 (Ar-$C_{5'}$), 128.48 (Ar-$C_{6'}$), 121.94 (q, $^1J_{F-C}$ = 271.7 Hz, CF_3), 100.95 (C-1), 70.09 (C-2), 73.11 (C-3), 68.12 (C-4), 75.99 (C-5), 60.36 (C-6) ppm. Anal. Calcd for $C_{13}H_{14}NO_8F_3$ (%): C, 42.27; H, 3.82; N, 3.79. Found: C, 42.26; H, 3.79; N, 3.78.

2-Chloro-3-trifluoromethylphenyl β-D-galactopyranoside **16** as white crystals, R_f 0.48 (1:9 MeOH/EtOAc), δ_H: 8.28 (1H, d, J = 2.0 Hz, Ar-H), 7.99 (1H, dd, J = 2.0, 9.2 Hz, Ar-H), 7.59 (1H, d, J = 8.8 Hz, Ar-H), 5.15 (1H, d, $J_{1,2}$ = 7.6 Hz, H-1), 3.55 (1H, dd, $J_{2,3}$ = 8.9 Hz, H-2), 3.50 (1H, dd, $J_{3,4}$ = 6.0 Hz, H-3), 3.47 (1H, d, $J_{4,5}$ = 6.0 Hz, H-4), 3.42 (1H, m, H-5), 3.67 (2H, m, H-6), 5.24 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 4.8 Hz, HO-2), 4.63 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 4.4 Hz, HO-3), 4.92 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 6.0 Hz, HO-4), 4.69 (1H, dd, $J_{H\text{-}6,OH\text{-}6}$ = 5.4, 5.6 Hz, HO-6) ppm; δ_C: 153.98 (Ar-$C_{1'}$), 120.13 (q, $^3J_{F-C}$ = 3.4 Hz, Ar-$C_{2'}$), 128.48 (q, $^2J_{F-C}$ = 30.1 Hz, Ar-$C_{3'}$), 131.62 (q, $^3J_{F-C}$ = 12.2 Hz, Ar-$C_{4'}$), 128.32 (Ar-$C_{5'}$), 131.56 (Ar-$C_{6'}$), 124.70 (q, $^1J_{F-C}$ = 260.0 Hz, CF_3), 100.95 (C-1), 69.97 (C-2), 73.29 (C-3), 68.03 (C-4), 76.04 (C-5), 60.30 (C-6) ppm. Anal. Calcd for $C_{13}H_{14}ClO_6F_3$ (%): C, 43.57; H, 3.94. Found: C, 43.55; H, 3.91.

2-Chloro-5-trifluoromethylphenyl β-D-galactopyranoside **17** as white crystals, R_f 0.50 (1:9 MeOH/EtOAc), δ_H: 7.69 (1H, d, J = 8.0 Hz, Ar-H), 7.55 (1H, d, J = 0.6 Hz, Ar-H), 7.59 (1H, dd, J = 2,1, 8.8 Hz, Ar-H), 5.11 (1H, d, $J_{1,2}$ = 8.4 Hz, H-1), 3.53 (1H, dd, $J_{2,3}$ = 10.2 Hz, H-2), 3.48 (1H, dd, $J_{3,4}$ = 5.2 Hz, H-3), 3.48 (1H, d, $J_{4,5}$ = 6.4 Hz, H-4), 3.44 (1H, m, H-5), 3.69 (2H, m, H-6), 5.20 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 3.6 Hz, HO-2), 4.58 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 2.8 Hz, HO-3), 4.91 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 3.6 Hz, HO-4), 4.65 (1H, dd, $J_{H\text{-}6,OH\text{-}6}$ = 5.0, 6.0 Hz, HO-6) ppm; δ_C: 153.01 (Ar-$C_{1'}$), 109.31 (Ar-$C_{2'}$), 130.97 (Ar-$C_{3'}$), 112.90 (q, $^3J_{F-C}$ = 2.8 Hz, Ar-$C_{4'}$), 122.32 (q, $^2J_{F-C}$ = 32.0 Hz,

Ar-$C_{5'}$), 121.42 (q, $^3J_{F-C}$ = 2.7 Hz, Ar-$C_{6'}$), 122.22 (q, $^1J_{F-C}$ = 262.0 Hz, CF_3), 100.81 (C-1), 70.02 (C-2), 73.31 (C-3), 68.02 (C-4), 75.73 (C-5), 60.18(C-6) ppm. Anal. Calcd for $C_{13}H_{14}ClO_6F_3$ (%): C, 43.57; H, 3.94. Found: C, 43.56; H, 3.93.

2-Trifluoromethylphenyl β-D-galactopyranoside **18** as white crystals, R_f 0.52 (1:9 MeOH/EtOAc), δ_H: 7.67 (1H, m, Ar-H), 7.60 (1H, dd, J = 5.2, 5.6 Hz, Ar-H), 7.33 (1H, d, J = 5.6 Hz, Ar-H), 7.12 (1H, dd, J = 4.8, 5.2 Hz, Ar-H), 5.00 (1H, d, $J_{1,2}$ = 7.8 Hz, H-1), 3.62 (1H, dd, $J_{2,3}$ = 10.8 Hz, H-2), 3.54 (1H, dd, $J_{3,4}$ = 4.8 Hz, H-3), 3.49 (1H, dd, $J_{4,5}$ = 5.4, 6.0 Hz, H-4), 3.40 (1H, m, H-5), 3.70 (2H, m, H-6), 4.97 (1H, d, $J_{H\text{-}2,OH\text{-}2}$ = 6.0 Hz, HO-2), 4.57 (1H, d, $J_{H\text{-}3,OH\text{-}3}$ = 4.2 Hz, HO-3), 4.91 (1H, d, $J_{H\text{-}4,OH\text{-}4}$ = 6.0 Hz, HO-4), 4.65 (1H, dd, $J_{H\text{-}6,OH\text{-}6}$ = 5.4, 6.5 Hz, HO-6) ppm; δ_C: 155.60 (Ar-$C_{1'}$), 131.65 (q, $^2J_{F-C}$ = 36.0 Hz, Ar-$C_{2'}$), 126.49 (q, $^3J_{F-C}$ = 3.6 Hz, Ar-$C_{3'}$), 121.14 (Ar-$C_{4'}$), 116.69 (Ar-$C_{5'}$), 133.95 (Ar-$C_{6'}$), 127.53 (q, $^1J_{F-C}$ = 208.4 Hz, CF_3), 100.46 (C-1), 70.13 (C-2), 73.58 (C-3), 68.04 (C-4), 75.62 (C-5), 60.30 (C-6) ppm. Anal. Calcd for $C_{13}H_{15}O_6F_3$ (%): C, 48.14; H, 4.67. Found: C, 48.12; H, 4.66.

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Synthesis and Characterization of Novel *lacZ* Gene Reporter Molecules: Detection of β-Galactosidase Activity by ^{19}F Nuclear Magnetic Resonance of Polyglycosylated Fluorinated Vitamin B_6

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Gene therapy has emerged as a promising strategy for treatment of various diseases. However, widespread implementation is hampered by difficulties in assessing the success of transfection, in particular, the spatial extent of expression in the target tissue and the longevity of expression. Thus, the development of noninvasive reporter techniques based on appropriate molecules and imaging modalities may help to assay gene expression. We have previously demonstrated the ability to detect β-galactosidase (β-gal) activity on the basis of ^{19}F NMR chemical shift associated with release of fluorophenyl aglycons from galactopyranoside conjugates. Use of fluoropyridoxol as the aglycon provides a potential less toxic alternative and we now report the design, synthesis, and structural analysis of a series of novel polyglycosylated fluorinated vitamin B_6 derivatives as ^{19}F NMR-sensitive aglycons for detection of *lacZ* gene expression. In particular, we report the activity of 3,α^4,α^5-tri-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol **4**, 3-*O*-(β-D-galactopyranosyl)-α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol **12**, and 3-*O*-(β-D-galactopyranosyl)-α^4,α^5-di-*O*-(α-D-mannopyranosyl)-6-fluoropyridoxol **13**. Compounds **4**, **12**, and **13** all show promising characteristics including highly sensitive ^{19}F NMR response to β-gal activity ($\Delta\delta = 9.0 \sim 9.4$ ppm), minimal toxicity for substrate or aglycon, and good water solubility. However, the differential glycosylation of **12** and **13** appears more advantageous for assessing *lacZ* gene expression in vivo.

Introduction

Gene therapy holds great promise for the treatment of diverse diseases, but widespread implementation is hindered by difficulties in assessing the success of transfection. The development of noninvasive in vivo reporter techniques based on appropriate molecules and imaging modalities would be of considerable value for assessing the location, magnitude, and persistence of expression.

The *lacZ* gene encoding β-galactosidase (β-gal) is widely used in molecular biology as a reporter gene to assay clonal insertion, transcriptional activation, protein expression, and protein interaction. Many colorimetric reporter molecules have been described to detect β-gal activity and these form the basis of highly effective spectrophotometric assays in vitro.[1−3] However, optical methods are less practical for applications in animals in vivo or ultimately in man in the clinic due to extensive light scattering and absorption by tissues. Toward such applications new reporter molecules are being developed. Recently, Tung et al.[4] presented a near-infrared approach in vivo based on 9*H*-(1,3-dichloro-9,9-dimethylacridin-2-on-7-yl) β-D-galactopyranoside to detect β-gal activity in transfected tumors in live mice. Lee et al.[5] described use of a radiolabeled competitive inhibitor 2-(4-[^{125}I/^{123}I]iodophenyl)ethyl 1-thio-β-D-galactopyranoside to detect β-gal activity in mice. Louie et al.[6] introduced an NMR approach using 1-[2-(β-D-galactopyranosyloxy)propyl]-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane)gadolinium(III) based on proton MRI contrast to detect β-gal activity in developing frog embryos following direct injection of substrate into eggs. We have been developing in vivo reporter molecules based on ^{19}F NMR with structures exploiting fluorophenol, trifluoromethanol, and fluoropyridoxol aglycons.[7−11] To date our published investigations have focused on development of reporter molecules and we have demonstrated detection of β-gal activity in cultured tumor cells with preliminary examples of detectability in tumors in living mice.[12] In a continuing effort to develop enhanced approaches for in vivo detection of β-gal, we now report the synthesis and evaluation of polyglycosylated fluorinated vitamin B_6 reporter molecules, designed to enhance water solubility, cellular penetration, and enzyme response.

Design

The diversity of substrates and reporter molecules for β-gal activity is indicative of broad substrate specificity. Agents have been tailored for specific imaging modalities or with particular characteristics, such as thermal stability suitable for autoclaving. However, some substrates suffer from poor aqueous solubility and inability to reach targets in vivo and some aglycon products are toxic. Our initial investigations used fluorophenyl β-D-galactopyranosides.[7,9] This approach was particularly facile, being a simple analogy of the classic "yellow" agent *o*-nitrophenyl β-D-galactopyranoside (ONPG). However, the product aglycon appears somewhat toxic, being closely similar to the uncoupler dinitrophenol. We were able to reduce the requisite concentration of reporter molecule by introducing a trifluoromethyl reporter moiety in place of a single fluorine atom, but this is characterized by a much smaller chemical shift response.[11] Toxicity could be largely avoided by using 6-fluoropyridoxol (**1**, FPOL) as the aglycon and we recently demonstrated proof of principle. Introduction of a D-galactose at the 3-phenolic group of FPOL, 3-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol (GFPOL), yielded a ^{19}F NMR gene expression reporter exhibiting a large chemical shift response to β-gal cleavage but having only moderate kinetic sensitivity to β-gal.[8] GFPOL was also modestly water-soluble.

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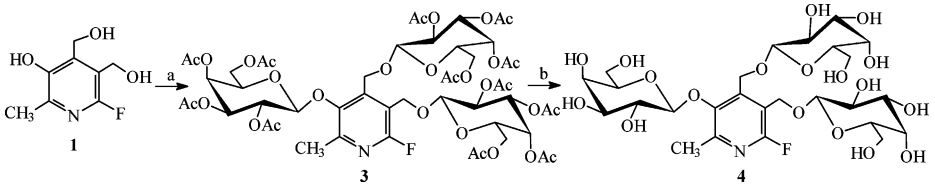

Figure 1. Reagents and conditions: (a) 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **2**, $Hg(CN)_2$, 4 Å molecular sieve, CH_2Cl_2, RT, 12 h, 89%; (b) NH_3/MeOH, 0 °C → RT, 24 h, quantitative yields.

We considered that introduction of additional sugar moieties could enhance water solubility and potentially improve enzyme sensitivity. Pertinent to this approach were reports that modification the α^4- and α^5-position hydroxymethyl moieties of FPOL produces modification of its pK_a with relatively minor changes in chemical shift and chemical shift range.[13] Further, *Escherichia coli* (*lacZ*) β-gal catalyzes the hydrolysis of galactopyranosides by cleavage of the C−O bond between D-galactose and the aglycon with a double-displacement mechanism involving the formation (glycosylation step) and breakdown (deglycosylation step) of a glycosyl-enzyme intermediate via oxocarbonium ion-like transition states. It has been observed that hydrogen-bonding interaction between the enzyme and the glycosidic substrate is important in the formation of the enzyme−substrate complex and the hydrolysis rate.[14,15] The involvement of fluorine atoms in hydrogen bonding is well documented and exemplified by some of the strongest known hydrogen bonds.[16] Considerable evidence suggests that a C−F moiety can act as a weak proton acceptor and may form hydrogen bonds between the enzyme and the substrate.[17−21]

We have found evidence of intramolecular hydrogen bonding between α^5-OH and 6-F in ^{1}H NMR spectra of FPOL and its derivatives. For example, the signal of α^5-OH in α^4-OH and α^5-OH unprotected analogues, such as FPOL or 3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyridoxol, always appears downfield and is coupled with 5-CH_2 as triplet due to the α^5-OH exchange limitation by the α^5-OH and 6-F hydrogen bonding. Meanwhile, α^4-OH occurs as an upfield singlet. Introduction of two additional carbohydrate residues at α^4- and α^5-hydroxymethyl positions of GFPOL would inhibit α^5-OH and 6-F hydrogen bonding and facilitate hydrogen bonding between the enzyme and the new substrates. Thus, substrate affinity should increase and both water solubility and enzyme sensitivity could be improved, while the virtues of GFPOL are retained.[8]

Results and Discussion

Syntheses. Our initial approach used a one-pot technique to introduce three D-galactose moieties at the 3 phenolic and α^4,α^5-hydroxymethylic sites, simultaneously. Reaction of **1** with 3.3 equiv of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **2** in anhydrous dichloromethane catalyzed by $Hg(CN)_2$ afforded the fully galactopyranosylated 6-fluoropyridoxol (**3**) in 89% yield, which was deacetylated with NH_3/MeOH, giving the free galactopyranoside 3,α^4,α^5-tri-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol **4** in quantitative yield (Figure 1). The ESI-MS of **3** showed the expected molecular ion at *m*/*z* 1178 and quasi-molecular ion at *m*/*z* 1179 [M + H], corresponding to the fully adorned derivative with three fully acetylated galactosides. The identity of **3** was established by ^{1}H and ^{13}C NMR. The anomeric protons H-1′, H-1″, and H-1‴ of D-galactoses linked to 3, α^4-, and α^5-positions of FPOL at 5.24, 4.66, and 4.52 ppm, respectively, with three well-resolved doublets ($J_{1,2}$ = 8.0 Hz), as well as $J_{2,3} \sim 10$ Hz, confirming that all D-galactoses are in the β-configuration with the $^4C_1$ chair conformation, whereas in the ^{13}C NMR spectrum, the anomeric carbons C-1′, C-1″, and C-1‴ occurred at 103.34 and 100.22 ppm.

Compound **4** was stable in buffer and gave a single sharp ^{19}F NMR signal. Exposure of **4** to β-gal indicated that all three β-D-galactopyranosyl $C_{1(gal)}$−O linkages are sensitive resulting in multiple ^{19}F signals around 3 ppm and 12∼20 ppm (Figure 2). These results demonstrate the principle of polyglycosylation to enhance water solubility while retaining sensitivity to β-gal, but the complex spectra suggested a need for a more sophisticated approach. We therefore designed two further molecules, 3-*O*-(β-D-galactopyranosyl)-α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol **12** and 3-*O*-(β-D-galactopyranosyl)-α^4,α^5-di-*O*-(α-D-mannopyranosyl)-6-fluoropyridoxol **13**, featuring differential glycosylation: galactosylation at the 3-phenolic group being sensitive to β-gal, and glucopyranosylation or mannopyranosylation at the α^4,α^5-hydroxymethyl groups to aid water solubility but resist β-gal activity. Retrosynthetic analysis suggested two approaches through differentially protected intermediates as key synthons.

6-Fluoro-α^4,α^5-isopropylidenepyridoxol **5** was previously prepared as part of the synthesis of 6-fluoro-3,α^4-isopropylidenepyridoxol.[8,13] Testing various acids as catalysts showed 2% H_2SO_4 acetone solution to provide the best yield of **5** (26%). The regioselectivity of the acetonation reaction was confirmed by comparing ^{1}H NMR of **5** and 6-fluoro-3,α^4-isopropylidenepyridoxol, in which the 5-CH_2 signal of **5** appeared at 5.03 ppm as a singlet, while in 6-fluoro-3,α^4-isopropylidenepyridoxol it appeared at 4.97 ppm as a doublet ($J_{H-5,HO-5}$ = 1.2 Hz) due to the coupling of 5-OH.[13] Treatment of **5** with **2** by the Koenigs−Knorr glycosylation gave 3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α^4,α^5-isopropylidene-6-fluoropyrodoxol **6** in 85% yield. The $\delta_{H-1'}$ at 4.64 ppm is a well-resolved doublet ($J_{1,2}$ = 8.0 Hz), and $\delta_{C-1'}$ at 100.03 ppm demonstrated that the D-galactose was in the β-configuration. The correlation between 2-CH_3 and H-1′ of sugar ring from the NOESY spectrum of **6** verified that 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl residue connected at the 3-phenolic site, providing further evidence that the acetonation had occurred regioselectively on 4,5-hydroxymethyl groups.

3-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyridoxol **7** was obtained by cleavage of acetonide **6**, but the yields were quite low (≤15%), based on several hydrolysis conditions, such as 80% AcOH, 1% HCl, or 90% CF_3CO_2H in MeOH, CH_2Cl_2, or 1,4-dioxane at various temperatures (60∼100 °C). A moderate amount of **1** was recoverable, indicating that the β-D-galactopyranosyl $C_{1'}$(gal)−O_3 bond became weak and sensitive to acid hydrolysis, presumably due to the presence of the 6-fluorine atom. Condensation of **7** with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** or 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide **9** in dry CH_2Cl_2 with $Hg(CN)_2$ as a promoter gave 3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyridoxol **10** or 3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol **11** in yields of 80% or 78%, respectively.

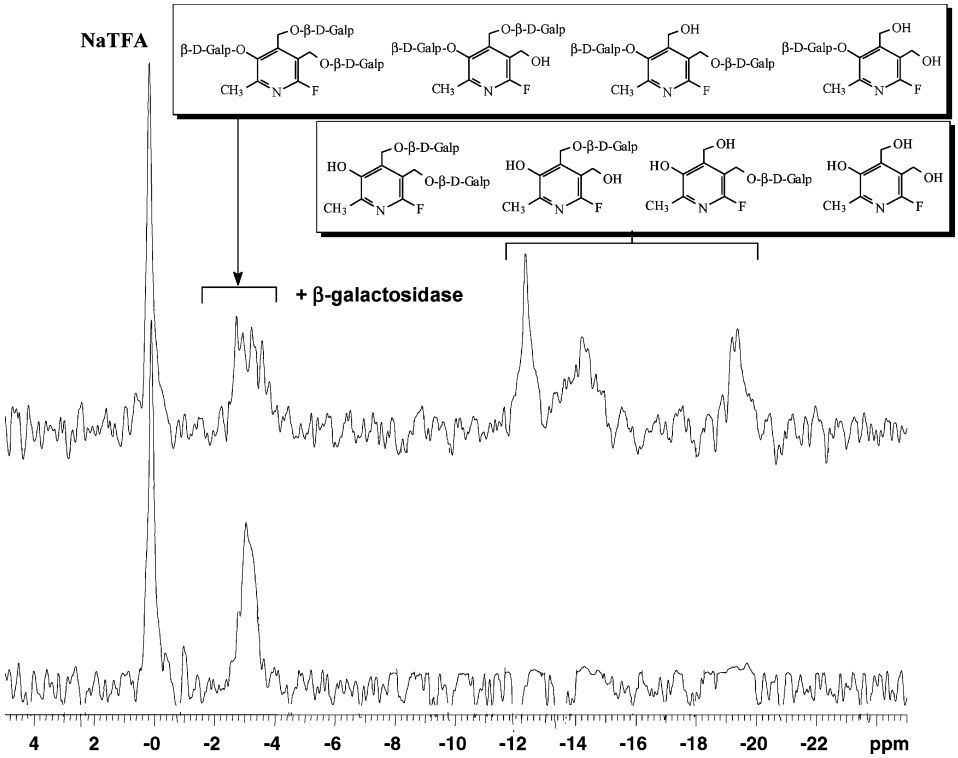

Figure 2. ^{19}F NMR spectra of 3,α^4,α^5-tri-*O*-(*β*-D-galactopyranosyl)-6-fluoropyridoxol **4** (10.1 mg, 15 mmol, lower trace) and its products resulting from addition of *β*-gal (E801A, 15 units) in PBS (pH = 7.4) at 37 °C (upper trace). Spectra were acquired in 51 s and enhanced with an exponential line broadening 40 Hz; *β*-D-Galp = *β*-D-galactopyranosyl.

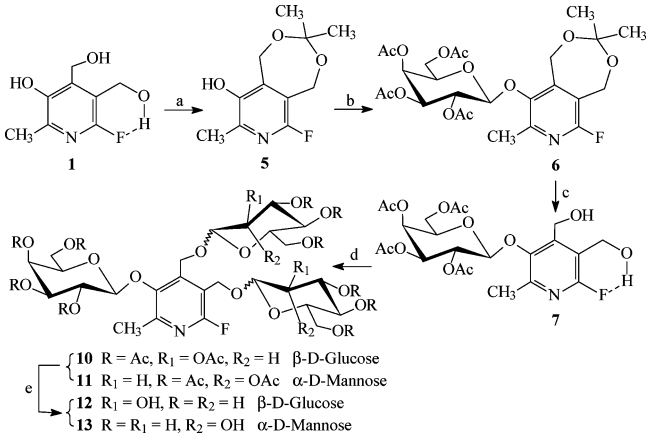

Figure 3. Reagents and conditions: (a) 2% H_2SO_4, acetone, RT 4∼5 h, 26%; (b) 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **2**, $Hg(CN)_2$, 4 Å molecular sieve, CH_2Cl_2, RT, 12 h, 85%; (c) 80% AcOH, 80 °C, 4∼5 h, 15%; (d) 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** or 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide **9**, $Hg(CN)_2$, 4 Å molecular sieve, CH_2Cl_2, RT, 12 h, 80% (→ **10**) or 78% (→ **11**), respectively; (e) NH_3/MeOH, 0 °C → RT, 24 h, quantitative yields.

Deacetylation of **10** or **11** in NH_3/MeOH from 0 °C to room temperature (RT) gave the target molecules **12** and **13** in quantitative yields (Figure 3). However, the overall yields for **12** and **13** through the five-step reactions were only 3%, with limiting steps in the α^4,α^5-isopropylidene group formation and hydrolysis procedures.

The acidic 3-phenolic group para to the 6-fluorine atom in FPOL should be easily converted into the monoanion under mild base conditions,[8,13,22] suggesting an alternate approach to selectively benzylate the 3-OH under carefully controlled conditions. Benzyl bromide (1.1 equiv) was added dropwise over a period of 4∼5 h to the well-stirred reaction mixture of **1** in a dichloromethane/aqueous biphasic system (pH 10∼11) with tetrabutylammonium bromide (TBAB) as the phase-transfer catalyst, yielding 3-*O*-benzyl-6-fluoropyridoxol **14** in 76% yield. The structure was established on the basis of the coupling characteristics of α^4,α^5-CH_2 as doublets ($J_{H-4,HO-4}$ = 6.0 Hz, $J_{H-5,HO-5}$ = 5.4 Hz) and α^4,α^5-OH as triplets in the ^1H NMR spectrum. Condensation of **14** with **8** or **9** gave 3-*O*-benzyl-α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-6-fluoropyridoxol **15** or 3-*O*-benzyl-α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol **16** in satisfactory yields. Removal of the benzyl-protecting group afforded acceptors α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl)-6-fluoropyridoxol **17** or α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol **18** in quantitative yields, which were subjected to a procedure similar to that described above for the preparation of galactosides, giving **10** or **11** in high yields (88% or 85%, respectively). After workup and deacetylation, the target compounds **12** and **13** were obtained in 57% and 52% overall yields over five-step reactions (Figure 4).

Recognizing the differential reactivity of the 3-phenolic group over the hydroxymethyl groups, most recently, we have successfully specifically galactopyranosylated **1** on the 3-phenolic group directly with **2** via the above phase-transfer catalysis technique, yielding **7**.[8] Figure 5 depicts a very efficient route to synthesize the target compounds **12** and **13** in just three steps with higher overall yields (67% and 65%, respectively).

Characteristics. Compounds **4**, **12**, and **13** each gave a single narrow ^{19}F NMR signal between *δ* −2.0 and −3.3 ppm, essentially invariant (Δ*δ* ≤ 0.06 ppm) with pH in the range 3−12 and temperatures from 25 to 37 °C in whole rabbit blood, 0.9% saline, or phosphate-buffered saline (PBS). Addition of *β*-gal (E801A) in PBS at 37 °C to **4**, **12** and **13** caused rapid hydrolysis, releasing the aglycons α^4,α^5-di-*O*-(*β*-D-galactopyranosyl)-6-fluoropyridoxol, α^4,α^5-di-*O*-(*β*-D-glucopyranosyl)-6-fluoropyridoxol, and α^4,α^5-di-*O*-(α-D-mannopyranosyl)-6-flu-

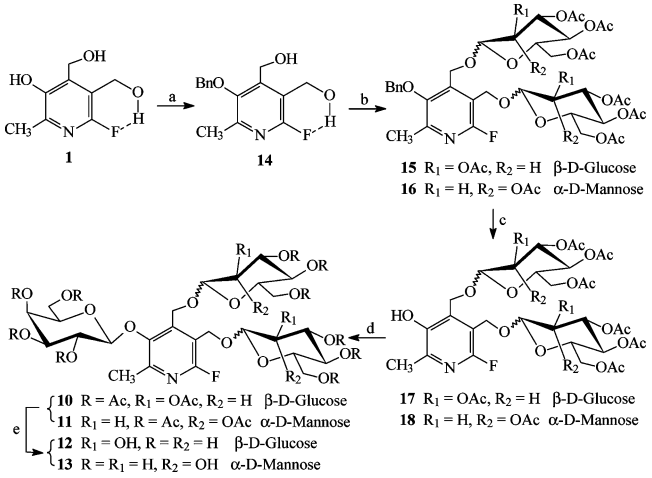

Figure 4. Reagents and conditions: (a) benzyl bromide (1.1 equiv), CH_2Cl_2−H_2O, pH 10∼11, 50 °C, TBAB, 4∼5 h, 76%; (b) 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** or 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide **9**, $Hg(CN)_2$, 4 Å molecular sieve, CH_2Cl_2, RT, 12 h, 90% (→ **15**) or 85% (→ **16**), respectively; (c) 25 psi H_2, Pd/C, RT, 12 h, quantitative yields; (d) 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **2**, $Hg(CN)_2$, 4 Å molecular sieve, CH_2Cl_2, RT, 12 h, 88% (→ **10**) or 85% (→ **11**), respectively; (e) NH_3/MeOH, 0 °C → RT, 24 h, 95% (→ **12**) or 94% (→ **13**), respectively.

oropyridoxol, which also appeared as single narrow ^{19}F signals between δ −11.20 and −12.40 ppm ($\Delta\delta$ = 9.0∼9.4 ppm) (Table 1). Action of β-gal on **4** was complicated by action on each of the galactose residues, apparently randomly, to generate multiple signals representing **1** together with partially galactosylated products (Figure 2). The β-gal hydrolysis of **4**, **12**, and **13** proceeded in a smooth manner, indicating that the liberated aglycons have no inhibitory effects on β-gal (Figure 6). The kinetic curves suggest straightforward first-order kinetics, which were much more rapid for all substrates than for GFPOL. Compounds **12** and **13** gave single products upon exposure to β-gal (Figure 7). Addition of **12** and **13** to stably transfected human breast MCF-7-*lacZ* tumor cells showed cleavage of **12** or **13** (Figure 8) and this proceeded in an initially smooth monotonic manner at rates of 18.6 or 19.6 μmol min^{-1} (million MCF7-*lacZ* cells)$^{-1}$, respectively. Compounds **12** and **13** have much higher aqueous solubility than GFPOL (GFPOL, 75 mM vs **12**, 196 mM, and **13**, 173 mM, all in PBS).

The products α^4,α^5-di-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol (DGFPOL), α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol (DUFPOL), and α^4,α^5-di-*O*-(α-D-mannopyranosyl)-6-fluoropyridoxol (DMFPOL) of the action of β-gal on **4**, **12**, and **13** also exhibit large ^{19}F NMR chemical shift response to pH ($\Delta\delta$ = ∼ 11.0 ppm) in the range of pH 1∼12 (Figure 9, Table 2), but there is no spectral overlap with the substrates.

Conclusion

These results provide further evidence for the broad specificity of β-gal and the feasibility of modifying substrate structures to enhance enzyme sensitivity and water solubility. The additional sugar residues in **4**, **12**, and **13** compared with GFPOL all lead to faster cleavage kinetics with β-gal. Significantly, the differential glycosylation provides structures that respond to β-gal with generation of single products. The results with stably transfected breast cancer cells indicate the potential for future studies in vivo.

Experimental Section

General Methods. NMR spectra were recorded on a Varian Inova 400 spectrometer (400 MHz for ^1H, 100 MHz for ^{13}C, 376 MHz for ^{19}F) with $CDCl_3$ or DMSO-d_6 as solvent. ^1H and ^{13}C chemical shifts are referenced to tetramethylsilane (TMS) as internal standard, and ^{19}F to a dilute solution of sodium trifluoroacetate (NaTFA) in a capillary as external standard (37 °C). Compounds were characterized by acquisition of ^1H, ^{13}C, distortionless enhancement by polarization transfer (DEPT), $^1H-^1H$ correlation spectroscopy (COSY), or nuclear Overhauser enhancement spectroscopy (NOESY) experiments at 25 °C. Microanalyses were performed on a Perkin-Elmer 2400CHN microanalyzer. Mass spectra were obtained by positive and negative electrospray ionization mass spectrometry (ESI-MS) on a Micromass Q-TOF hybrid quadrupole/time-of-flight instrument (Micromass UK Ltd.). Reactions requiring anhydrous conditions were performed under nitrogen or argon. $Hg(CN)_2$ was dried before use at 50 °C for 1 h, CH_2Cl_2 was dried over Drierite, and acetonitrile was dried on CaH_2 and kept over molecular sieves under N_2. Solutions in organic solvents were dried with anhydrous sodium sulfate and concentrated in vacuo below 45 °C. 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide **2** and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** were purchased from Sigma Chemical Co. 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl bromide **9** was prepared according to the literature method.[23] Column chromatography was performed on silica gel (200∼300 mesh) by elution with cyclohexane/EtOAc, and silica gel GF_{254} (Aldrich) was used for analytical thin-layer chromatography (TLC). Detection was effected by spraying the plates with 5% ethanolic H_2SO_4 (followed by heating at 110 °C for ∼10 min) or by direct UV illumination of the plate.

For enzyme kinetic experiments, **4**, **12**, and **13** (10.1 mg, 15 mmol) were dissolved in PBS (0.1 M, pH = 7.4, 600 μL), and a PBS solution of β-gal (0.1 M, pH = 7.4, 15 μL, 1 unit/μL, E801A, Promega, Madison, WI) was added and NMR data were acquired immediately at 37 °C.

MCF7-*lacZ* human breast cancer cells stably transfected to express β-gal were grown in culture under standard conditions and harvested. Compound **12** or **13** was added to suspension of cells (5×10^6) in PBS and observed by NMR for 1 h.

Syntheses: 3,α^4,α^5-Tri-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyridoxol 3. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **2** (1.35 g, 3.3 mmol, 1.1 equiv) in anhydrous CH_2Cl_2 (8 mL) was added dropwise to a solution of 6-fluoropyridoxol **1** (0.18 g, 1.0 mmol) and $Hg(CN)_2$ (1.01 g, 4.0 mmol) in dry MeCN (10 mL) containing powdered molecular sieves (4 Å, 2.0 g) with vigorous stirring at RT under argon in the dark for 12 h. The mixture was diluted with CH_2Cl_2 (30 mL), filtered through Celite, washed, dried (Na_2SO_4), and concentrated in vacuo. The residue was purified on a silica gel column (1:3 cyclohexane/EtOAc) to yield **3** (1.05 g, 89%) as syrup, R_f 0.30 (1:3 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 5.24 (1H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.04 (1H, dd, $J_{2',3'}$ = 9.8 Hz, H-2′), 4.73 (1H, dd, $J_{3',4'}$ = 3.4 Hz, H-3′), 3.98 (1H, dd, $J_{4',5'}$ = 2.4 Hz, H-4′), 4.02∼4.10 (3H, m, H-5′ and H-6′), 4.66 (1H, d, $J_{1'',2''}$ = 8.0 Hz, H-1″), 4.52 (1H, d, $J_{1''',2'''}$ = 8.0 Hz, H-1‴), 5.15 (2H, dd, $J_{2'',3''}$ = $J_{2''',3'''}$ = 10.0 Hz, H-2″ and H-2‴), 5.07 (2H, dd, $J_{3'',4''}$ = $J_{3''',4'''}$ = 3.6 Hz, H-3″ and H-3‴), 5.52 (2H, dd, $J_{4'',5''}$ = $J_{4''',5'''}$ = 3.2 Hz, H-4″ and H-4‴), 3.88 (2H, m, H-5″ and H-5‴), 4.18 (2H, dd, $J_{5'',6a''}$ = $J_{5''',6a'''}$ = 3.6 Hz, $J_{6a'',6b''}$ = $J_{6a''',6b'''}$ = 9.2 Hz, H-6a″ and H-6a‴), 4.11 (2H, dd, $J_{5'',6b''}$ = $J_{5''',6b'''}$ = 6.8 Hz, H-6b″ and H-6b‴), 4.48 (2H, d, $J_{CH2-4a,CH2-4b}$ = $J_{CH2-5a,CH2-5b}$ = 13.2 Hz, CH_2-4a and CH_2-5a), 4.12 (2H, d, $J_{CH2-4a,CH2-4b}$ = $J_{CH2-5a,CH2-5b}$ = 13.2 Hz, CH_2-4b and CH_2-5b), 2.43 (3H, s, CH_3-2), 2.18, 2.17, 2.16, 2.15, 2.12, 2.11, 2.10, 2.09, 2.08, 2.07, 2.06, 2.05 (36H, 12s, 12 CH_3CO). δ_C 170.84, 170.79, 170.77, 170.73, 170.68, 170.54, 170.53, 170.49, 170.45, 170.35, 170.31, 170.28 (12 CH_3**C**O), 146.47 (d, $^3J_{F-C}$ = 14.5 Hz, Py-C_2), 148.16 (d, $^4J_{F-C}$ = 3.8 Hz, Py-C_3), 133.10 (s, Py-C_4), 112.51 (d, $^2J_{F-C}$ = 31.3 Hz, Py-C_5), 155.04 (d, $^1J_{F-C}$ = 231.2 Hz, Py-C_6), 103.34 (s, C-1′), 100.22 (s, C-1″ and

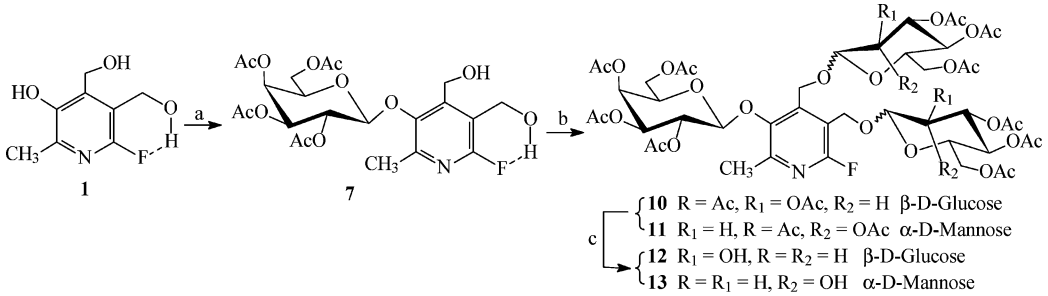

Figure 5. Reagents and conditions: (a) 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide **2**, $CH_2Cl_2-H_2O$, pH 10∼11, RT, TBAB, 4∼5 h, 88%; (b) 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** or 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide **9**, $Hg(CN)_2$, 4 Å molecular sieve, CH_2Cl_2, RT, 12 h, 80% (→ **10**) or 78% (→ **11**), respectively; (c) NH_3/MeOH, 0 °C → RT, 24 h, 95% (→ **12**) or 94% (→ **13**), respectively.

Table 1. ^{19}F Chemical Shifts[a] and Hydrolytic Rates[b]

reporters	4	12	13	GFPOL
$\delta_{F(substrate)}$	−3.02	−2.85	−2.14	−3.22
$\delta_{F(product)}$	−12.37	−12.16	−11.22	−11.21
$\Delta\delta_F$	9.35	9.31	9.08	7.99
v (μmol min^{-1} unit^{-1})	34.0	35.0	38.0	4.3

[a] Chemical shifts are given in parts per million (ppm) with respect to sodium trifluoroacetate. [b] β-gal (E801A) was added at 37 °C in PBS (0.1 M, pH = 7.4).

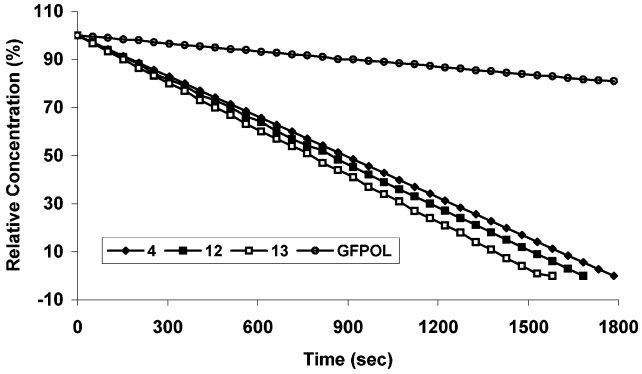

Figure 6. Kinetic hydrolysis time courses of **4** (◆), **12** (■), **13** (□) (15.0 mmol each), and GFPOL (○) (10.0 mmol) by β-gal (E801A, 15 units) hydrolysis in PBS (0.1 M, pH = 7.4, 600 μL) at 37 °C.

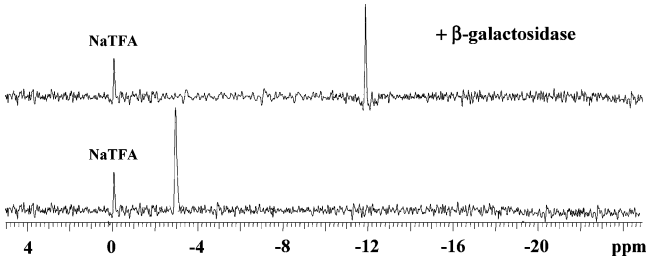

Figure 7. ^{19}F NMR spectra of 3-*O*-(β-D-galactopyranosyl)-α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol **12** (10.1 mg, 15 mmol, lower trace) and its hydrolysis by β-gal (E801A, 15 units) in PBS (0.1 M, pH = 7.4, 600 μL) at 37 °C (upper trace). Spectra were acquired in 205 s and enhanced with exponential line broadening = 40 Hz.

C-1‴), 70.75 (s, C-2′), 71.14 (s, C-3′), 70.61 (s, C-4′), 71.56 (s, C-5′), 67.03 (s, C-6′), 67.45 (s, C-2″ and C-2‴), 68.39 (s, C-3″ and C-3‴), 66.31 (s, C-4″ and C-4‴), 68.55 (s, C-5″ and C-5‴), 61.99 (s, C-6″ and C-6‴), 61.54 (s, CH_2-4), 61.67 (s, CH_2-5), 21.03, 20.94, 20.90, 20.89, 20.87, 20.85, 20.83, 20.79, 20.77, 20.76, 20.74, 20.72 (12s, 12 CH_3CO), 18.77 (s, CH_3-3). ESI-MS *m*/*z* 1178 [M$^+$] (26%), 1179 [M + 1] (14%). Anal. Calcd for $C_{50}H_{64}NO_{30}F$: C, 50.96, H, 5.48, N, 1.19. Found: C, 50.93, H, 5.46, N, 1.15.

3,α^4,α^5-Tri-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol 4. A solution of **3** (0.9 g) in anhydrous MeOH (20 mL) containing 0.5 M NH_3 was vigorously stirred from 0 °C to RT overnight, until TLC showed complete reaction, and then evaporated to dryness in vacuo. Chromatography of the crude syrup on silica gel with EtOAc/MeOH (4:1) afforded **4** (0.52 g) as a syrup in quantitative yield, R_f 0.10 (1:4 MeOH/EtOAc). NMR (DMSO-d_6) δ_H 4.95 (1H, d, $J_{1',2'}$ = 8.2 Hz, H-1′), 4.76 (1H, dd, $J_{2',3'}$ = 10.0 Hz, H-2′), 4.91 (1H, dd, $J_{3',4'}$ = 2.8 Hz, H-3′), 5.11 (1H, dd, $J_{4',5'}$ = 2.3 Hz, H-4′), 3.77 (1H, m, H-5′), 3.90 (1H, dd, $J_{5',6a'}$ = 6.4 Hz, $J_{6a',6b'}$ = 12.4 Hz,

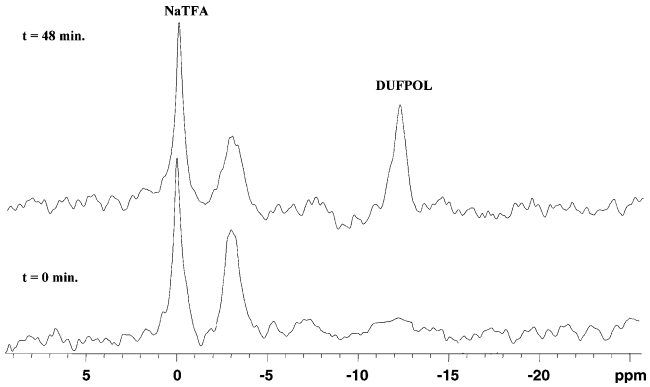

Figure 8. ^{19}F NMR spectra of 3-*O*-(β-D-galactopyranosyl)-α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol **12** (5.1 mg, 7.5 mmol) with stably transfected MCF7-*lacZ* cells (5 × 10^6) in PBS (0.1 M, pH = 7.4, 600 μL) at 37 °C. Spectra were acquired in 51 s and enhanced with an exponential line broadening = 100 Hz. [DUFPOL = α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol.]

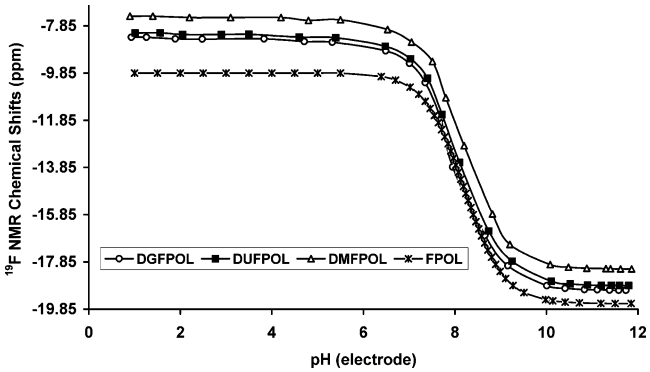

Figure 9. ^{19}F NMR chemical shift pH titration curve of DGFPOL, DUFPOL, and DMFPOL in 0.9% saline at 37 °C. [DGFPOL = α^4,α^5-di-*O*-(β-D-galactopyranosyl)-6-fluoropyridoxol; DUFPOL = α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol; DMFPOL = α^4,α^5-di-*O*-(α-D-mannopyranosyl)-6-fluoropyridoxol.]

Table 2. Acidities and ^{19}F NMR/pH Properties of DGFPOL, DUFPOL, DMFPOL, and FPOL in Saline at 25 °C[a]

pH indicators	DGFPOL	DUFPOL	DMFPOL	FPOL[24]
pK_a	7.95	8.08	8.18	8.20
δ_{Facid}	−8.34	−8.15	−7.44	−9.85
δ_{Fbase}	−19.05	−18.85	−18.15	−19.61

[a] Chemical shifts are given in parts per million (ppm) with respect to sodium trifluoroacetate.

H-6a′), 3.68 (1H, dd, $J_{5',6b'}$ = 3.6 Hz, H-6b′), 4.22 (2H, d, $J_{1'',2''}$ = $J_{1''',2'''}$ = 8.0 Hz, H-1″ and H-1‴), 3.29 (2H, dd, $J_{2'',3''}$ = $J_{2''',3'''}$ = 10.6 Hz, H-2″ and H-2‴), 3.51 (2H, dd, $J_{3'',4''}$ = $J_{3''',4'''}$ = 3.2 Hz, H-3″ and H-3‴), 3.62 (2H, dd, $J_{4'',5''}$ = $J_{4''',5'''}$ = 2.4 Hz, H-4″ and H-4‴), 3.46 (2H, m, H-5″ and H-5‴), 3.66 (2H, dd, $J_{5'',6a''}$ = $J_{5''',6a'''}$ = 3.6 Hz, $J_{6a'',6b''}$ = $J_{6a''',6b'''}$ = 10.4 Hz, H-6a″ and H-6a‴), 3.39 (2H, dd, $J_{5'',6b''}$ = $J_{5''',6b'''}$ = 6.6 Hz, H-6b″ and H-6b‴), 4.48 (2H, d, $J_{CH2-4a,CH2-4b}$ = $J_{CH2-5a,CH2-5b}$ = 13.0 Hz, CH_2-4a and CH_2-5a), 4.44 (2H, d, $J_{CH2-4a,CH2-4b}$ = $J_{CH2-5a,CH2-5b}$ = 13.0 Hz, CH_2-4b and CH_2-5b), 2.32 (3H, s, CH_3-2). δ_C 144.65 (d, $^3J_{F-C}$ = 14.5 Hz, Py-C_2), 147.87 (d, $^4J_{F-C}$ = 3.9 Hz, Py-C_3), 137.36 (d, $^4J_{F-C}$ = 3.8 Hz, Py-C_4), 115.15 (d, $^2J_{F-C}$ = 32.3 Hz, Py-C_5), 154.26 (d, $^1J_{F-C}$ = 226.6 Hz, Py-C_6), 103.19 (s, C-1′), 101.67 (s, C-1″ and C-1‴), 70.36 (s, C-2′), 73.94 (s, C-3′), 69.44 (s, C-4′), 76.08 (s, C-5′), 62.88 (s, C-6′), 72.10 (s, C-2″ and C-2‴), 73.50 (s, C-3″ and C-3‴), 68.26 (s, C-4″ and C-4‴), 75.02 (s, C-5″ and C-5‴), 60.60 (s, C-6″ and C-6‴), 68.77 (s, CH_2-4), 68.92 (s, CH_2-5), 19.19 (s, CH_3-3). ESI-MS *m/z* 673 [M^+] (6%), 674 [M + 1] (10%). Anal. Calcd for $C_{26}H_{40}NO_{18}F$: C, 46.34, H, 5.99, N, 2.08. Found: C, 46.30, H, 5.96, N, 2.05.

α⁴,α⁵-*O*-Isopropylidene-6-fluoropyridoxol 5. A suspension of **1** (0.50 g, 2.67 mmol) in anhydrous acetone (40 mL) containing 2% concentrated H_2SO_4 was stirred for 4∼5 h, at the end of which time TLC (4:1 cyclohexane/EtOAc) indicated complete reaction, and then cold saturated Na_2CO_3 solution was added with vigorous stirring up to pH between 8 and 9. The precipitate was filtered off, and concentration of the reaction mixture under reduced pressure followed by purification on flash silica gel column (4:1 cyclohexane/EtOAc) gave **5** (0.64 g, 26%) as a syrup, R_f 0.34 (4:1 cyclohexane/EtOAc), NMR ($CDCl_3$) δ_H 7.45 (1H, s, HO-3), 5.03 (2H, s, CH_2-5), 4.57 (2H, s, CH_2-4), 2.33 (3H, s, CH_3-2), 1.55 (6H, s, 2 CH_3). δ_C 146.40 (d, $^3J_{F-C}$ = 14.5 Hz, Py-C_2), 144.32 (d, $^4J_{F-C}$ = 3.8 Hz, Py-C_3), 130.68 (s, Py-C_4), 111.21 (d, $^2J_{F-C}$ = 32.8 Hz, Py-C_5), 152.20 (d, $^1J_{F-C}$ = 231.2 Hz, Py-C_6), 100.15 (s, **C**Me_2), 58.70 (d, $^3J_{F-C}$ = 3.0 Hz, CH_2-5), 54.51 (s, CH_2-4), 31.62 [s, C(**C**H_3)$_2$], 17.58 (s, CH_3-2). Anal. Calcd for $C_{11}H_{14}NO_3F$: C, 58.13, H, 6.21, N, 6.17. Found: C, 58.08, H, 6.16, N, 6.11.

3-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-α⁴,α⁵-*O*-isopropylidene-6-fluoropyridoxol 6. To a solution of **5** (0.62 g, 2.72 mmol) and $Hg(CN)_2$ (0.88 g, 3.50 mmol) in dry CH_2Cl_2 (10 mL) containing freshly activated 4 Å molecular sieves (2.0 g) was added dropwise compound **2** (1.23 g, 3.0 mmol, 1.1 equiv). The mixture was stirred overnight in the dark at RT under N_2 until TLC indicated complete reaction. Workup as for **3** gave **6** (1.29 g, 85%), R_f 0.40 (2:3 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 4.64 (1H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.25 (1H, dd, $J_{2',3'}$ = 10.0 Hz, H-2′), 5.02 (1H, dd, $J_{3',4'}$ = 3.6 Hz, H-3′), 5.41 (1H, dd, $J_{4',5'}$ = 3.2 Hz, H-4′), 3.97 (1H, m, H-5′), 4.21 (1H, dd, $J_{5',6a'}$ = 4.4 Hz, $J_{6a',6b'}$ = 11.2 Hz, H-6a′), 4.13 (1H, dd, $J_{5',6b'}$ = 7.2 Hz, H-6b′), 5.10 (1H, d, $J_{CH2-4a,CH2-4b}$ = 8.0 Hz, CH_2-4a), 4.67 (1H, d, $J_{CH2-4a,CH2-4b}$ = 8.0 Hz, CH_2-4b), 5.14 (1H, d, $J_{CH2-5a,CH2-5b}$ = 9.6 Hz, CH_2-5a), 5.12 (1H, d, $J_{CH2-5a,CH2-5b}$ = 9.6 Hz, CH_2-5b), 2.42 (3H, s, CH_3-2), 2.17, 2.09, 2.08, 1.99 (12H, 4s, 4 CH_3CO), 1.61, 1.59 (6H, 2s, 2 CH_3). δ_C 170.78, 170.39, 170.26, 170.11 (4s, 4 CH_3**C**O), 145.48 (d, $^3J_{F-C}$ = 15.2 Hz, Py-C_2), 133.16 (d, $^4J_{F-C}$ = 4.0 Hz, Py-C_3), 126.26 (s, Py-C_4), 116.95 (d, $^2J_{F-C}$ = 32.1Hz, Py-C_5), 154.30 (d, $^1J_{F-C}$ = 229.0 Hz, Py-C_6), 101.41 (s, **C**Me_2), 100.03 (s, C-1′), 68.70 (s, C-2′), 70.82 (s, C-3′), 67.12 (s, C-4′), 71.53 (s, C-5′), 64.28 (s, C-6′), 55.38 (s, CH_2-4), 61.58 (s, CH_2-5), 31.88 (s, C(**C**H_3)$_2$), 20.90, 20.89, 20.82, 20.77 (4s, 4 CH_3CO), 18.77 (s, CH_3-2). Anal. Calcd for $C_{25}H_{32}NO_{12}F$: C, 53.84, H, 5.79, N, 2.51. Found: C, 53.79, H, 5.74, N, 2.49.

3-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-6-fluoropyridoxol 7. A mixture of **6** (1.25 g, 2.50 mmol) in 80% AcOH (40 mL) was stirred at 80 °C for 4∼5 h, till TLC (1:3 cyclohexane/EtOAc) showed complete reaction. The cooled mixture was neutralized with cold saturated Na_2CO_3 solution, extracted with EtOAc (4 × 30 mL), and concentrated and purified by flash silica gel column with 1:4 cyclohexane/EtOAc, giving **7** (0.17 g, 15%) as a syrup, R_f 0.18 (1:4 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 4.79 (1H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.55 (1H, dd, $J_{2',3'}$ = 10.6 Hz, H-2′), 5.10 (1H, dd, $J_{3',4'}$ = 3.6 Hz, H-3′), 5.41 (1H, dd, $J_{4',5'}$ = 3.6 Hz, H-4′), 3.88 (1H, m, H-5′), 4.24 (1H, dd, $J_{5',6a'}$ = 4.4 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6a′), 4.09 (1H, dd, $J_{5',6b'}$ = 6.0 Hz, H-6b′), 5.01 (2H, d, $J_{CH2-4a,CH2-4b}$ = $J_{CH2-5a,CH2-5b}$ = 12.4 Hz, CH_2-4a and CH_2-5a), 4.62 (1H, d, $J_{CH2-4a,CH2-4b}$ = 12.4 Hz, CH_2-4b), 4.66 (1H, d, $J_{CH2-5a,CH2-5b}$ = 12.4 Hz, CH_2-5b), 3.50 (1H, m, α⁴-HO, exchangeable with D_2O), 3.56 (1H, m, α⁵-HO, exchangeable with D_2O), 2.47 (3H, s, CH_3-2), 2.23, 2.17, 2.02, 2.00 (12H, 4s, 4 CH_3CO). δ_C 170.32, 170.28, 170.18, 169.48 (4 CH_3**C**O), 150.33 (d, $^3J_{F-C}$ = 15.2 Hz, Py-C_2), 147.62 (d, $^4J_{F-C}$ = 4.6 Hz, Py-C_3), 146.32 (d, $^3J_{F-C}$ = 4.5 Hz, Py-C_4), 120.17 (d, $^2J_{F-C}$ = 32.0 Hz, Py-C_5), 157.60 (d, $^1J_{F-C}$ = 235.8 Hz, Py-C_6), 102.39 (s, C-1′), 68.91 (s, C-2′), 70.74 (s, C-3′), 67.19 (s, C-4′), 71.93 (s, C-5′), 61.98 (s, C-6′), 55.91 (s, **C**H_2-4), 59.60 (s, **C**H_2-5), 20.99, 20.85, 20.70, 20.67 (4s, 4 CH_3CO), 19.46 (s, CH_3-2). Anal. Calcd for $C_{22}H_{28}NO_{12}F$: C, 51.05, H, 5.46, N, 2.71. Found: C, 51.00, H, 5.39, N, 2.68.

Alternately **7** was synthesized from **1** directly by phase transfer catalysis: to a well-stirred CH_2Cl_2 (10 mL)/H_2O (10 mL) biphasic mixture (pH 10∼11) of **1** (0.5 g, 2.67 mmol) and TBAB (0.1 g, 0.31 mmol), a solution of **2** (1.21 g, 2.94 mmol, 1.1 equiv) in CH_2Cl_2 (10 mL) was added dropwise over a period of 4∼5 h at RT, and the stirring continued for an additional hour. The products were extracted (EtOAc; 4 × 20 mL), washed free of alkali, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel (1:4 cyclohexane/EtOAc) to afford **7** (1.08 g, 88%) as syrup, which is identical in all respects to the product obtained above.

3-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-α⁴,α⁵-di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyridoxol 10 and 3-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-α⁴,α⁵-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol 11. Condensation of **7** (0.5 g, 1.1 mmol) with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **8** or 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide **9** (1.0 g, 2.40 mmol, 1.1 equiv) in dry CH_2Cl_2 (10 mL) with $Hg(CN)_2$ (0.63 g, 2.50 mmol) as a promoter, according to the procedures described for the preparation of **3** and **6**, furnished 3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α⁴,α⁵-di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyridoxol **10** and 3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α⁴,α⁵-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol **11**, respectively.

3-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-α⁴,α⁵-di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyridoxol 10: 1.04 g, 80%, syrup, R_f 0.30 (1:3 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 5.06 (1H, d, $J_{1',2'}$ = 7.8 Hz, H-1′), 5.28 (1H, dd, $J_{2',3'}$ = 8.8 Hz, H-2′), 4.98 (1H, dd, $J_{3',4'}$ = 4.8 Hz, H-3′), 4.73 (1H, dd, $J_{4',5'}$ = 2.8 Hz, H-4′), 3.95 (1H, m, H-5′), 4.19 (1H, dd, $J_{5',6a'}$ = 3.6 Hz, $J_{6a',6b'}$ = 10.8 Hz, H-6a′), 4.02 (1H, dd, $J_{5',6b'}$ = 5.2 Hz, H-6b′), 5.36 (1H, d, $J_{1'',2''}$ = 8.0 Hz, H-1″), 5.39 (1H, d, $J_{1''',2'''}$ = 8.0 Hz, H-1‴), 5.12 (1H, dd, $J_{2'',3''}$ = 7.2 Hz, H-2″), 5.15 (1H, dd, $J_{2''',3'''}$ = 6.8 Hz, H-2‴), 5.04 (1H, dd, $J_{3'',4''}$ = 3.2 Hz, H-3″), 5.07 (1H, dd, $J_{3''',4'''}$ = 3.6 Hz, H-3‴), 4.76 (1H, dd, $J_{4'',5''}$ = 2.8 Hz, H-4″), 4.78 (1H, dd, $J_{4''',5'''}$ = 2.8 Hz, H-4‴), 3.91 (1H, m, H-5″), 3.93 (1H, m, H-5‴), 4.08 (1H, dd, $J_{5'',6a''}$ = 3.2 Hz, $J_{6a'',6b''}$ = 9.4 Hz, H-6a″), 4.10 (1H, dd, $J_{5''',6a'''}$ = 3.0 Hz, $J_{6a''',6b'''}$ = 10.0 Hz, H-6a‴), 4.04 (1H, dd, $J_{5'',6b''}$ = 7.6 Hz, H-6b″), 4.07 (1H, dd, $J_{5''',6b'''}$ = 6.8 Hz, H-6b‴), 4.55 (2H, d, $J_{CH2-4a,CH2-4b}$ = $J_{CH2-5a,CH2-5b}$ = 11.2 Hz, CH_2-4a and CH_2-5b), 4.49 (1H, d, $J_{CH2-4a,CH2-4b}$ = 11.2 Hz, CH_2-4b), 4.91 (1H, d, $J_{CH2-5a,CH2-5b}$ = 11.2 Hz, CH_2-5a), 2.34 (3H, s, CH_3-2), 2.05, 1.98, 1.97, 1.96, 1.95, 1.94, 1.93, 1.92, 1.91, 1.90, 1.89, 1.88 (36H, 12s, 12 CH_3CO). δ_C 170.83, 170.80, 170.76, 170.72, 170.70, 170.56, 170.28, 170.20, 170.17, 170.00, 169.82, 169.75 (12 CH_3CO), 152.14 (d, $^3J_{F-C}$ = 16.0 Hz, Py-C_2), 149.81 (s, Py-C_3), 138.42 (d, $^3J_{F-C}$ = 11.4 Hz, Py-C_4), 117.48 (d, $^2J_{F-C}$ = 32.0 Hz, Py-C_5), 157.56 (d, $^1J_{F-C}$ = 233.5 Hz, Py-C_6), 102.66 (s, C-1′), 98.00 (s, C-1″), 98.06 (s, C-1‴), 71.09 (s, C-2′), 68.65 (s, C-2″), 68.95 (s, C-2‴), 74.46 (s, C-3′), 70.84 (s, C-3″), 71.51 (s, C-3‴), 70.05 (s, C-4′), 68.13 (s, C-4″), 68.22 (s, C-4‴), 75.10 (s, C-5′), 72.20 (s, C-5″), 74.24 (s, C-5‴), 63.75 (s, C-6′), 61.91 (s, C-6″), 63.86 (s, C-6‴), 56.77 (s, CH_2-4), 57.16 (s, CH_2-5), 21.20, 20.95, 20.93, 20.91, 20.89, 20.87, 20.85, 20.75, 20.67, 20.62, 20.58,

20.54 (12s, 12 CH_3CO), 19.76 (s, CH_3-3). ESI-MS *m*/*z* 1178 [M^+] (28%), 1179 [M + 1] (12%). Anal. Calcd for $C_{50}H_{64}NO_{30}F$: C, 50.96, H, 5.48, N, 1.19. Found: C, 50.92, H, 5.44, N, 1.16.

3-*O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol 11: 1.01 g, 78%, syrup, R_f 0.35 (1:3 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 4.80 (1H, d, $J_{1',2'}$ = 8.2 Hz, H-1′), 5.13 (1H, dd, $J_{2',3'}$ = 9.8 Hz, H-2′), 5.36 (1H, dd, $J_{3',4'}$ = 4.2 Hz, H-3′), 5.30 (1H, dd, $J_{4',5'}$ = 3.6 Hz, H-4′), 4.01 (1H, m, H-5′), 4.33 (1H, dd, $J_{5',6a'}$ = 3.2 Hz, $J_{6a',6b'}$ = 10.0 Hz, H-6a′), 4.11 (1H, dd, $J_{5',6b'}$ = 4.6 Hz, H-6b′), 4.71 (2H, d, $J_{1'',2''} = J_{1''',2'''}$ = 2.4 Hz, H-1″ and H-1‴), 4.74 (2H, dd, $J_{2'',3''} = J_{2''',3'''}$ = 6.2 Hz, H-2″ and H-2‴), 5.22 (2H, dd, $J_{3'',4''} = J_{3''',4'''}$ = 3.8 Hz, H-3″ and H-3‴), 3.95 (2H, dd, $J_{4'',5''} = J_{4''',5'''}$ = 2.0 Hz, H-4″ and H-4‴), 4.02 (2H, m, H-5″ and H-5‴), 4.11 (2H, dd, $J_{5'',6a''} = J_{5''',6a'''}$ = 2.0 Hz, $J_{6a'',6b''} = J_{6a''',6b'''}$ = 7.4 Hz, H-6a″ and H-6a‴), 4.07 (2H, dd, $J_{5'',6b''} = J_{5''',6b'''}$ = 5.6 Hz, H-6b″ and H-6b‴), 4.87 (2H, d, $J_{CH2-4a,CH2-4b} = J_{CH2-5a,CH2-5b}$ = 13.6 Hz, CH_2-4a and CH_2-5b), 4.67 (1H, d, $J_{CH2-4a,CH2-4b} = J_{CH2-5a,CH2-5b}$ = 13.6 Hz, CH_2-4b and CH_2-5a), 2.33 (3H, s, CH_3-2), 2.07, 2.04, 2.03, 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94, 1.93, 1.92 (36H, 12s, 12 CH_3CO). δ_C 171.27, 171.23, 171.15, 171.06, 170.87, 170.83, 170.76, 170.63, 170.58, 170.44, 170.29, 170.25 (12 CH_3**C**O), 153.06 (d, $^3J_{F-C}$ = 16.0 Hz, Py-C_2), 149.41 (d, $^4J_{F-C}$ = 4.6 Hz, Py-C_3), 145.38 (d, $^3J_{F-C}$ = 4.6 Hz, Py-C_4), 117.21 (d, $^2J_{F-C}$ = 31.3 Hz, Py-C_5), 159.55 (d, $^1J_{F-C}$ = 235.0 Hz, Py-C_6), 103.62 (s, C-1′), 98.32 (s, C-1″), 98.61 (s, C-1‴), 70.75 (s, C-2′), 70.22 (s, C-2″), 70.26 (s, C-2‴), 71.83 (s, C-3′), 70.36 (s, C-3″), 70.39 (s, C-3‴), 69.38 (s, C-4′), 67.04 (s, C-4″), 68.60 (s, C-4‴), 72.54 (s, C-5′), 71.90 (s, C-5″), 72.45 (s, C-5‴), 61.47 (s, C-6′), 62.46 (s, C-6″), 63.53 (s, C-6‴), 56.44 (s, CH_2-4), 56.46 (s, CH_2-5), 21.29, 21.21, 21.19, 21.17, 21.13, 21.10, 21.08, 21.05, 21.00, 20.95, 20.90, 20.88 (12s, 12 CH_3CO), 20.35 (s, CH_3-3). ESI-MS *m*/*z* 1178 [M^+] (20%), 1179 [M + 1] (17%). Anal. Calcd for $C_{50}H_{64}NO_{30}F$: C, 50.96, H, 5.48, N, 1.19. Found: C, 50.94, H, 5.45, N, 1.16.

3-*O*-(β-D-Galactopyranosyl)-α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol 12 and 3-*O*-(β-D-Galactopyranosyl)-α^4,α^5-di-*O*-(α-D-mannopyranosyl)-6-fluoropyridoxol 13. Compounds **10** and **11** (1.00 g, 0.85 mmol) were deacetylated as described above for **4** to yield **12** and **13** in quantitative yields.

3-*O*-(β-D-Galactopyranosyl)-α^4,α^5-di-*O*-(β-D-glucopyranosyl)-6-fluoropyridoxol 12: 0.57 g, foam solid, R_f 0.20 (1:4 MeOH/EtOAc). NMR (DMSO-d_6) δ_H 5.01 (1H, d, $J_{1',2'}$ = 8.2 Hz, H-1′), 5.22 (1H, dd, $J_{2',3'}$ = 9.0 Hz, H-2′), 4.92 (1H, dd, $J_{3',4'}$ = 4.6 Hz, H-3′), 4.70 (1H, dd, $J_{4',5'}$ = 2.6 Hz, H-4′), 3.91 (1H, m, H-5′), 4.12 (1H, dd, $J_{5',6a'}$ = 3.2 Hz, $J_{6a',6b'}$ = 10.2 Hz, H-6a′), 4.00 (1H, dd, $J_{5',6b'}$ = 5.6 Hz, H-6b′), 5.14 (2H, d, $J_{1'',2''}$ = 10.0 Hz, H-1″ and H-1‴), 4.82 (2H, dd, $J_{2'',3''} = J_{2''',3'''}$ = 8.2 Hz, H-2″ and H-2‴), 4.69 (2H, dd, $J_{3'',4''} = J_{3''',4'''}$ = 3.4 Hz, H-3″ and H-3‴), 4.93 (2H, dd, $J_{4'',5''} = J_{4''',5'''}$ = 3.2 Hz, H-4″ and H-4‴), 3.65 (2H, m, H-5″ and H-5‴), 3.55 (2H, dd, $J_{5'',6a''} = J_{5''',6a'''}$ = 4.8 Hz, $J_{6a'',6b''} = J_{6a''',6b'''}$ = 12.0 Hz, H-6a″ and H-6a‴), 3.31 (2H, dd, $J_{5'',6b''} = J_{5''',6b'''}$ = 5.6 Hz, H-6b″ and H-6b‴), 4.29 (1H, d, $J_{CH2-4a,CH2-4b}$ = 7.6 Hz, CH_2-4a), 4.36 (1H, d, $J_{CH2-4a,CH2-4b}$ = 7.6 Hz, CH_2-5a), 4.20 (2H, d, $J_{CH2-4a,CH2-4b} = J_{CH2-5a,CH2-5b}$ = 7.6 Hz, CH_2-4b and CH_2-5b), 4.18∼3.65 (12H, br, HO-2′, 3′, 4′, 6′, 2″, 3″, 4″, 6″, 2‴, 3‴, 4‴, and 6‴, exchangeable with D_2O), 2.42 (3H, s, CH_3-2). δ_C 144.43 (d, $^3J_{F-C}$ = 15.0 Hz, Py-C_2), 136.26 (d, $^4J_{F-C}$ = 3.8 Hz, Py-C_3), 124.40 (d, $^3J_{F-C}$ = 3.8 Hz, Py-C_4), 120.39 (d, $^2J_{F-C}$ = 32.8 Hz, Py-C_5), 148.98 (d, $^1J_{F-C}$ = 259.7 Hz, Py-C_6), 103.65 (s, C-1′), 101.76 (s, C-1″ and C-1‴), 72.34 (s, C-2′), 71.28 (s, C-2″ and C-2‴), 74.55 (s, C-3′), 73.88 (s, C-3″ and C-3‴), 69.82 (s, C-4′), 68.89 (s, C-4″ and C-4‴), 76.62 (s, C-5′), 77.29 (s, C-5″ and C-5‴), 61.36 (s, C-6′), 60.95 (s, C-6″ and C-6‴), 60.54 (s, CH_2-4), 60.78 (s, CH_2-5), 19.88 (s, CH_3-3). ESI-MS *m*/*z* 673 [M^+] (8%), 674 [M + 1] (14%). Anal. Calcd for $C_{26}H_{40}NO_{18}F$: C, 46.34, H, 5.99, N, 2.08. Found: C, 46.32, H, 5.97, N, 2.07.

3-*O*-(β-D-Galactopyranosyl)-α^4,α^5-di-*O*-(α-D-mannopyranosyl)-6-fluoropyridoxol 13: 0.57 g, foam solid, R_f 0.26 (1:4 MeOH/EtOAc). NMR (DMSO-d_6) δ_H 5.00 (1H, d, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.23 (1H, dd, $J_{2',3'}$ = 10.0 Hz, H-2′), 5.16 (1H, dd, $J_{3',4'}$ = 3.8 Hz, H-3′), 5.08 (1H, dd, $J_{4',5'}$ = 3.2 Hz, H-4′), 4.21 (1H, m, H-5′), 4.51 (1H, dd, $J_{5',6a'}$ = 3.6 Hz, $J_{6a',6b'}$ = 10.2 Hz, H-6a′), 4.31 (1H, dd, $J_{5',6b'}$ = 4.8 Hz, H-6b′), 4.84 (2H, d, $J_{1'',2''} = J_{1''',2'''}$ = 2.6 Hz, H-1″ and H-1‴), 4.68 (2H, dd, $J_{2'',3''} = J_{2''',3'''}$ = 6.0 Hz, H-2″ and H-2‴), 5.02 (2H, dd, $J_{3'',4''} = J_{3''',4'''}$ = 3.6 Hz, H-3″ and H-3‴), 4.05 (2H, dd, $J_{4'',5''} = J_{4''',5'''}$ = 2.2 Hz, H-4″ and H-4‴), 3.94 (2H, m, H-5″ and H-5‴), 4.21 (2H, dd, $J_{5'',6a''} = J_{5''',6a'''}$ = 2.4 Hz, $J_{6a'',6b''} = J_{6a''',6b''}$ = 8.4 Hz, H-6a″ and H-6a‴), 4.17 (2H, dd, $J_{5'',6b''} = J_{5''',6b'''}$ = 6.5 Hz, H-6b″ and H-6b‴), 4.77 (2H, d, $J_{CH2-4a,CH2-4b} = J_{CH2-5a,CH2-5b}$ = 11.6 Hz, CH_2-4a and CH_2-5b), 4.57 (1H, d, $J_{CH2-4a,CH2-4b} = J_{CH2-5a,CH2-5b}$ = 11.6 Hz, CH_2-4b and CH_2-5a), 2.45 (3H, s, CH_3-2), 4.30∼3.70 (12H, br, HO-2′, 3′, 4′, 6′, 2″, 3″, 4″, 6″, 2‴, 3‴, 4‴, and 6‴, exchangeable with D_2O). δ_C 151.07 (d, $^3J_{F-C}$ = 15.3 Hz, Py-C_2), 148.61 (d, $^4J_{F-C}$ = 4.8 Hz, Py-C_3), 144.27 (d, $^3J_{F-C}$ = 3.6 Hz, Py-C_4), 116.29 (d, $^2J_{F-C}$ = 32.0 Hz, Py-C_5), 157.25 (d, $^1J_{F-C}$ = 233.5 Hz, Py-C_6), 103.68 (s, C-1′), 98.56 (s, C-1″), 98.68 (s, C-1‴), 71.76 (s, C-2′), 70.66 (s, C-2″), 70.86 (s, C-2‴), 72.38 (s, C-3′), 71.46 (s, C-3″), 71.32 (s, C-3‴), 70.48 (s, C-4′), 66.87 (s, C-4″), 67.90 (s, C-4‴), 73.64 (s, C-5′), 72.20 (s, C-5″), 72.65 (s, C-5‴), 62.77 (s, C-6′), 63.56 (s, C-6″), 64.83 (s, C-6‴), 57.54 (s, CH_2-4), 58.41 (s, CH_2-5), 20.12 (s, CH_3-3). ESI-MS *m*/*z* 673 [M^+] (5%), 674 [M + 1] (9%). Anal. Calcd for $C_{26}H_{40}NO_{18}F$: C, 46.34, H, 5.99, N, 2.08. Found: C, 46.31, H, 5.97, N, 2.05.

3-*O*-Benzyl-6-fluoropyridoxol 14. To a well-stirred CH_2Cl_2 (10 mL)/H_2O (10 mL) biphasic mixture (pH 10∼11) of **1** (0.50 g, 2.67 mmol) and TBAB (0.10 g, 0.31 mmol), a solution of benzyl bromide (0.51 g, 2.94 mmol, 1.1 equiv) in CH_2Cl_2 (10 mL) was added dropwise over a period of 4∼5 h, while the reaction temperature was maintained at 50 °C, and the stirring continued for an additional hour. Products were extracted (CH_2Cl_2, 4 × 20 mL), washed free of alkali, dried (Na_2SO_4), and concentrated, and the residue was purified by column chromatography on silica gel with 1:2 cyclohexane/EtOAc to afford major product **14** (0.56 g, 76%), white crystalline, R_f 0.38 (1:2 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 7.39 (5H, m, Ar-H), 4.90 (2H, s, $PhCH_2$), 4.75 (2H, d, $J_{H-5,HO-5}$ = 5.4 Hz, CH_2-5), 4.72 (2H, d, $J_{H-4,HO-4}$ = 6.0 Hz, CH_2-4), 3.57 (1H, t, $J_{H-5,HO-5}$ = 5.4 Hz, α^5-OH, exchangeable with D_2O), 3.49 (1H, t, $J_{H-4,HO-4}$ = 6.0 Hz, α^4-OH, exchangeable with D_2O), 2.44 (3H, s, CH_3-2). δ_C 151.34 (d, $^3J_{F-C}$ = 9.6 Hz, Py-C_2), 146.97 (d, $^4J_{F-C}$ = 2.9 Hz, Py-C_3), 149.55 (d, $^3J_{F-C}$ = 3.1 Hz, Py-C_4), 119.09 (d, $^2J_{F-C}$ = 20.8 Hz, Py-C_5), 156.30 (d, $^1J_{F-C}$ = 216.2 Hz, Py-C_6), 136.33, 128.96, 128.88, 128.57 (Ph-C), 55.99 (s, $PhCH_2$, CH_2-4), 56.76 (s, CH_2-5), 19.31 (s, CH_3-2). Anal. Calcd for $C_{15}H_{16}NO_3F$: C, 64.96, H, 5.82, N, 5.05. Found: C, 64.95, H, 5.79, N, 5.04.

3-*O*-Benzyl-α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyridoxol 15 and 3-*O*-Benzyl-α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol 16. Glycosylation of **14** (0.46 g, 2.0 mmol) with **8** or **9** (1.83 g, 4.45 mmol, 1.1 equiv) was carried out as for **3**, **10**, and **11** to give **15** and **16**, respectively.

3-*O*-Benzyl-α^4,α^5-di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyridoxol 15: 0.32 g, 95%) syrup, R_f 0.35 (3:2 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 7.41 (5H, m, Ar-H), 5.36 (1H, d, $J_{1',2'}$ = 8.2 Hz, H-1′), 5.41 (1H, d, $J_{1'',2''}$ = 8.2 Hz, H-1″), 5.14 (2H, dd, $J_{2',3'} = J_{2'',3''}$ = 7.4 Hz, H-2′ and H-2″), 4.45 (2H, dd, $J_{3',4'} = J_{3'',4''}$ = 3.3 Hz, H-3′ and H-3″), 4.84 (2H, dd, $J_{4',5'} = J_{4'',5''}$ = 3.8 Hz, H-4′ and H-4″), 3.96 (2H, m, H-5′ and H-5″), 4.80 (2H, dd, $J_{5',6a'} = J_{5'',6a''}$ = 2.6 Hz, $J_{6a',6b'} = J_{6a'',6b''}$ = 10.1 Hz, H-6a′ and H-6a″), 4.10 (2H, dd, $J_{5',6b'} = J_{5'',6b''}$ = 3.0 Hz, H-6b′ and H-6b″), 4.94 (2H, s, $PhCH_2$), 4.55 (1H, d, $J_{CH2-4a,CH2-4b}$ = 10.4 Hz, CH_2-4a), 4.48 (1H, d, $J_{CH2-4a,CH2-4b}$ = 10.4 Hz, CH_2-4b), 4.60 (1H, d, $J_{CH2-5a,CH2-5b}$ = 11.0 Hz, CH_2-5a), 4.52 (1H, d, $J_{CH2-5a,CH2-5b}$ = 11.0 Hz, CH_2-5b), 2.37 (3H, s, CH_3-2), 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94, 1.93 (24H, 8s, 8 CH_3CO). δ_C 170.84, 170.76, 170.31, 170.29, 170.26, 169.95, 169.92, 169.84 (8 CH_3**C**O), 152.18 (d, $^3J_{F-C}$ = 14.5 Hz, Py-C_2), 142.64 (d, $^4J_{F-C}$ = 4.6 Hz, Py-C_3), 150.12 (d, $^3J_{F-C}$ = 3.8 Hz, Py-C_4), 116.20 (d, $^2J_{F-C}$ = 32.0 Hz, Py-C_5), 157.40 (d, $^1J_{F-C}$ = 234.3 Hz, Py-C_6), 136.37, 129.00, 128.94, 128.87, 128.16, 127.77 (Ph-C), 100.23 (s, C-1′), 100.41 (s, C-1″), 71.41 (s, C-2′ and C-2″), 72.08 (s, C-3′), 72.19 (s, C-3″), 68.34 (s, C-4′), 68.51 (s, C-4″), 72.86 (s, C-5′), 72.93 (s, C-5″),

61.86 (s, C-6′), 61.98 (s, C-6″), 60.98 (s, CH_2-4), 61.28 (s, CH_2-5), 20.88, 20.85, 20.82, 20.75, 20.73, 20.60, 20.59, 20.58 (8s, 8 CH_3CO), 19.43 (s, CH_3-3). ESI-MS *m/z* 937 [M^+] (35%), 938 [M + 1] (25%). Anal. Calcd for $C_{43}H_{52}NO_{21}F$: C, 55.05, H, 5.59, N, 1.49. Found: C, 55.03, H, 5.57, N, 1.48.

3-*O*-Benzyl-α⁴,α⁵-di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol 16: 0.30 g, 90%, syrup, R_f 0.40 (3:2 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 7.38 (5H, m, Ar-H), 5.38 (1H, d, $J_{1',2'} = 2.6$ Hz, H-1′), 5.41 (1H, d, $J_{1'',2''} = 2.6$ Hz, H-1″), 5.36∼3.95 (18H, m, H-2′, 3′, 4′, 5′, 6′, 2″, 3″, 4″, 5″, 6″, $PhCH_2$, CH_2-4, and CH_2-5), 2.38 (3H, s, CH_3-2), 2.02, 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94 (24H, 8s, 8 CH_3CO). δ_C 171.25, 171.18, 170.89, 170.85, 170.78, 170.66, 170.60, 170.48 (8 $CH_3\mathbf{C}O$), 153.28 (d, $^3J_{F-C} = 15.8$ Hz, Py-C_2), 145.48 (d, $^4J_{F-C} = 4.8$ Hz, Py-C_3), 150.16 (d, $^3J_{F-C} = 3.8$ Hz, Py-C_4), 116.30 (d, $^2J_{F-C} = 31.0$ Hz, Py-C_5), 157.77 (d, $^1J_{F-C} = 206.8$ Hz, Py-C_6), 98.42 (s, C-1′), 100.03 (s, C-1″), 72.60∼56.54 (13C, C-2′, 3′, 4′, 5′, 6′, 2″, 3″, 4″, 5″, 6″, $Ph\mathbf{C}H_2$, CH_2-4, and CH_2-5), 21.23, 20.94, 20.92, 20.90, 20.88, 20.86, 20.84, 20.80, 20.78 (8s, 8 CH_3CO), 18.37 (s, CH_3-3). ESI-MS *m/z* 937 [M^+] (32%), 938 [M + 1] (20%). Anal. Calcd for $C_{43}H_{52}NO_{21}F$: C, 55.05, H, 5.59, N, 1.49. Found: C, 55.01, H, 5.55, N, 1.45.

α⁴,α⁵-Di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyridoxol 17 and α⁴,α⁵-Di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol 18. A mixture of **15** or **16** (0.29 g, 0.30 mmol) and Pd−C (5%, 50 mg) in MeOH (40 mL) was stirred for 24 h at RT under H_2 (25 psi). Evaporated filtrate gave **17** and **18** in quantitative yields.

α⁴,α⁵-Di-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-6-fluoropyridoxol 17: 0.26 g, syrup, R_f 0.28 (1:3 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 7.33 (1H, s, HO-3, exchangeable with D_2O), 5.30 (1H, d, $J_{1',2'} = 8.4$ Hz, H-1′), 5.35 (1H, d, $J_{1'',2''} = 8.4$ Hz, H-1″), 5.09 (2H, dd, $J_{2',3'} = J_{2'',3''} = 7.6$ Hz, H-2′ and H-2″), 4.35 (2H, dd, $J_{3',4'} = J_{3'',4''} = 3.4$ Hz, H-3′ and H-3″), 4.80 (2H, dd, $J_{4',5'} = J_{4'',5''} = 3.6$ Hz, H-4′ and H-4″), 3.89 (2H, m, H-5′ and H-5″), 4.77 (2H, dd, $J_{5',6a'} = J_{5'',6a''} = 2.4$ Hz, $J_{6a',6b'} = J_{6a'',6b''} = 10.6$ Hz, H-6a′ and H-6a″), 4.05 (2H, dd, $J_{5',6b'} = J_{5'',6b''} = 3.2$ Hz, H-6b′ and H-6b″), 4.51 (1H, d, $J_{CH2-4a,CH2-4b} = 10.3$ Hz, CH_2-4a), 4.45 (1H, d, $J_{CH2-4a,CH2-4b} = 10.3$ Hz, CH_2-4b), 4.57 (1H, d, $J_{CH2-5a,CH2-5b} = 11.1$ Hz, CH_2-5a), 4.49 (1H, d, $J_{CH2-5a,CH2-5b} = 11.1$ Hz, CH_2-5b), 2.35 (3H, s, CH_3-2), 1.99, 1.98, 1.97, 1.96, 1.95, 1.94, 1.93, 1.91 (24H, 8s, 8 CH_3CO). δ_C 170.82, 170.78, 170.65, 170.58, 170.46, 169.85, 169.82, 169.80 (8 $CH_3\mathbf{C}O$), 152.28 (d, $^3J_{F-C} = 14.2$ Hz, Py-C_2), 148.28 (d, $^4J_{F-C} = 3.2$ Hz, Py-C_3), 142.69 (d, $^3J_{F-C} = 4.8$ Hz, Py-C_4), 116.26 (d, $^2J_{F-C} = 32.2$ Hz, Py-C_5), 157.56 (d, $^1J_{F-C} = 231.4$ Hz, Py-C_6), 100.35 (s, C-1′), 100.54 (s, C-1″), 71.37 (s, C-2′ and C-2″), 72.18 (s, C-3′), 72.29 (s, C-3″), 68.38 (s, C-4′), 68.56 (s, C-4″), 72.83 (s, C-5′), 72.88 (s, C-5″), 61.82 (s, C-6′), 61.89 (s, C-6″), 60.90 (s, CH_2-4), 61.19 (s, CH_2-5), 20.85, 20.83, 20.82, 20.80, 20.78, 20.76, 20.73, 20.65 (8s, 8 CH_3CO), 19.32 (s, CH_3-3). ESI-MS *m/z* 847 [M^+] (30%), 848 [M + 1] (21%). Anal. Calcd for $C_{36}H_{46}NO_{21}F$: C, 50.99, H, 5.47, N, 1.65. Found: C, 50.96, H, 5.45, N, 1.62.

α⁴,α⁵-Di-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-6-fluoropyridoxol 18: 0.26 g, syrup, R_f 0.27 (1:3 cyclohexane/EtOAc). NMR ($CDCl_3$) δ_H 7.33 (1H, s, HO-3, exchangeable with D_2O), 5.33 (1H, d, $J_{1',2'} = 2.7$ Hz, H-1′), 5.37 (1H, d, $J_{1'',2''} = 2.7$ Hz, H-1″), 5.45∼4.07 (16H, m, H-2′, 3′, 4′, 5′, 6′, 2″, 3″, 4″, 5″, 6″, CH_2-4, and CH_2-5), 2.35 (3H, s, CH_3-2), 2.01, 2.00, 1.99, 1.98, 1.97, 1.96, 1.95, 1.94 (24H, 8s, 8 CH_3CO); δ_C: 171.33, 171.21, 170.85, 170.83, 170.76, 170.61, 170.56, 170.53 (8 $CH_3\mathbf{C}O$), 153.67 (d, $^3J_{F-C} = 15.8$ Hz, Py-C_2), 149.08 (d, $^4J_{F-C} = 3.0$ Hz, Py-C_3), 145.68 (d, $^3J_{F-C} = 4.6$ Hz, Py-C_4), 118.23 (d, $^2J_{F-C} = 31.2$ Hz, Py-C_5), 157.59 (d, $^1J_{F-C} = 223.1$ Hz, Py-C_6), 98.67 (s, C-1′), 100.33 (s, C-1″), 72.8∼56.56 (12C, C-2′, 3′, 4′, 5′, 6′, 2″, 3″, 4″, 5″, 6″, CH_2-4, and CH_2-5), 20.99, 20.97, 20.93, 20.90, 20.88, 20.86, 20.84, 20.80 (8s, 8 CH_3CO), 18.45 (s, CH_3-3). ESI-MS *m/z* 847 [M^+] (25%), 848 [M + 1] (18%). Anal. Calcd for $C_{36}H_{46}NO_{21}F$: C, 50.99, H, 5.47, N, 1.65. Found: C, 50.97, H, 5.44, N, 1.63.

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Imaging β-galactosidase activity using ^{19}F chemical shift imaging of LacZ gene-reporter molecule 2-fluoro-4-nitrophenol-β-D-galactopyranoside

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Abstract

2-Fluoro-4-nitrophenol-β-D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluoroaryl-β-D-galactopyranosides), which are highly responsive to the action of β-galactosidase (β-gal). OFPNPG has a single ^{19}F peak (55 ppm relative to aqueous sodium trifluoroacetate). Upon cleavage by β-gal, the pH sensitive aglycone 2-fluoro-4-nitrophenol (OFPNP) is observed at a chemical shift of 59–61 ppm. The chemical shift response is sufficient to observe β-gal activity using chemical shift imaging (CSI). ^{19}F CSI studies of enzyme activity and *lacZ* gene expression in 9L-glioma and MCF7 breast cancer cells are presented, providing further evidence for the utility of OFPNPG as a gene-reporter molecule for future in vivo studies.

Keywords: β-Galactosidase; ^{19}F NMR; CSI; *lacZ* gene reporter; Breast cancer; Phenylgalactopyranosides

1. Introduction

Although gene therapy has great potential for the treatment of diverse diseases, its widespread implementation is hindered by difficulties in assessing the success of transfection in terms of spatial extent, gene expression, and longevity of expression. Strategies for identifying exogenous gene activity have been presented using radionuclide imaging [1,2], optical imaging [3,4] and NMR [5,6].

β-Galactosidase (β-gal), the product of the lacZ gene, was the first expression system to be identified and characterized some 50 years ago, and it has become a fundamental tool in molecular biology as a reporter gene. Diverse colorimetric substrates have been developed suitable for in vitro or histological assays of β-gal [7]. More recently, Louie et al. [8] presented a proton MRI contrast agent, Tung et al. [9] used a near infrared active substrate and Lee et al. [10] reported a radioiodinated substrate to detect the activity of β-gal. An alternate strategy uses ^{19}F-labeled molecules as NMR active substrates, thus exploiting the high NMR visibility of fluorine, the great NMR sensitivity of ^{19}F to the environmental milieu and the lack of background signal [11]. We have recently demonstrated the feasibility of using ^{19}F NMR to detect chemical shift changes accompanying β-gal-induced cleavage of the prototype reporter molecule, 2-nitro-4-fluorophenyl β-D-galactopyranoside (PFONPG) [12].

2-Fluoro-4-nitrophenyl-beta-D-galactopyranoside (OFPNPG) is an isomer of PFONPG, which is highly responsive to the action of β-gal enzyme [13]. The molecule is stable in solution and with respect to wild-type cells, but β-gal causes rapid liberation of the aglycone 2-fluoro-4-nitrophenol (OFPNP), which has a pH-dependent ^{19}F NMR chemical shift, 4–6 ppm upfield from OFPNPG. We have chosen to develop imaging approaches using OFPNPG rather than PFONPG, since the aglycone appears to be less toxic and the pK_a is outside the normal physiological range. We now present ^{19}F NMR chemical shift imaging (CSI) studies of the conversion of OFPNPG to OFPNP β-gal enzyme in solution and lacZ transfected cancer cells lines.

2. Experimental

Human MCF7 breast cancer cells were stably transfected with recombinant vector phCMV/*lacZ* using

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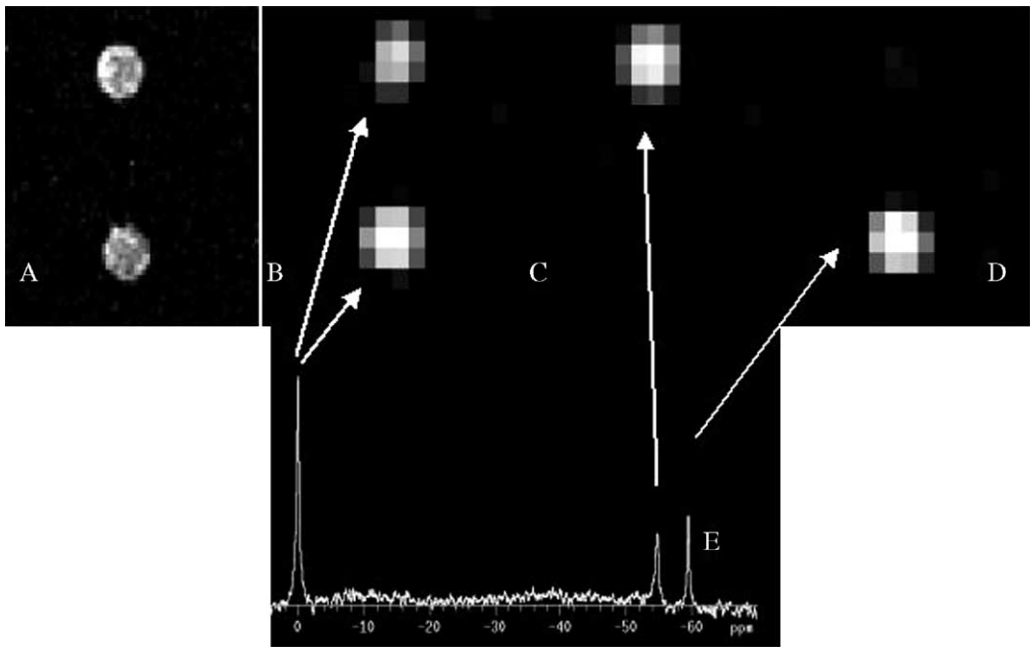

Fig. 1. ^{19}F CSI of OFPNPG and OFPNP. Two vials containing Na-TFA (75 mM) and OFPNPG (70 mM) or OFPNP (70 mM), respectively, were imaged by CSI: (A) spin echo proton scout image, ^{19}F CSI images of (B) Na-TFA, (C) OFPNPG and (D) OFPNP. (E) The corresponding bulk spectrum (with 30 Hz line broadening).

GenePORTER2 (Gene Therapy Systems), inserting the *E. coli lacZ* gene (from pSV-β-gal vector, Promega) under control of the high expression human cytomegalovirus (CMV) immediate-early enhancer/promoter vector phCMV (Gene Therapy Systems). Clonal selection was applied to identify those MCF-7 cells with the highest β-gal expression and these were grown in culture dishes under standard conditions and harvested [14]. 9L-Glioma cells stably transfected to express lacZ were kindly provided by Dr. Steven Brown (Henry Ford Hospital, Detroit, MI, USA). Imaging experiments used a Varian INOVA Unity 4.7-T system (188.2 MHz for ^{19}F) with a standard 2D spin echo CSI sequence to image the conversion of OFPNPG to OFPNP. MRI parameters were as follows: field of view= 30×30 mm, spectral window=70 ppm, slice thickness= 10 mm, matrix=16×16, TR/TE= 1000/12 ms. Chemical shift imaging data were reconstructed and analyzed with homebuilt programs written using the MATLAB programming language. Sodium trifluoroacetate (Na-TFA) (10 mg/ml) was used as internal standard. OFPNPG was dissolved in phosphate buffered saline (PBS) to yield a 70 mM solution, which was used in all experiments. For CSI studies with

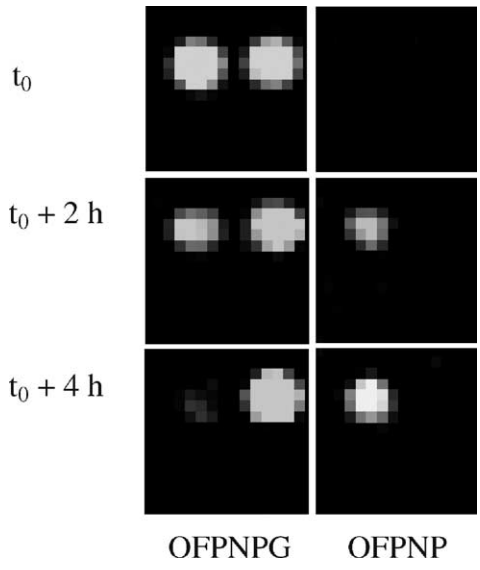

Fig. 2. Conversion of OFPNPG to OFPNP by β-gal enzyme. Two vials contained OFPNPG (70 mM). Upon addition of β-gal to the left vial, the intensity of OFPNPG signal was found to decrease, while that of OFPNP increased (right panel). Each image was acquired in 4½ min.

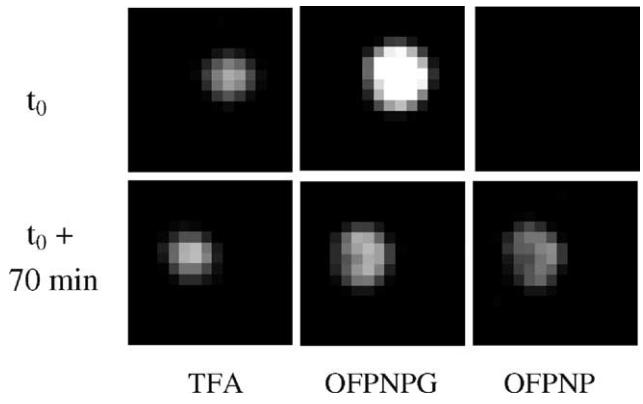

Fig. 3. Conversion of OFPNPG to OFPNP by 9L-lacZ rat glioma cells. 10^8 cells were added to a vial containing OFPNPG (70mM) and imaged. ^{19}F CSI revealed the conversion of OFPNPG to OFPNP over 70 min.

β-gal enzyme, a PBS solution of β-gal (180 μl, G-2513, 0.22 unit/μl, Aldrich Chemical) was added to an aliquot of this solution and ^{19}F CSI data were acquired over 4 h at the ambient magnet bore temperature (18°C). For CSI studies with tumor cells, 10^7 9L-lacZ or 10^8 MCF7-lacZ cells were added to the solution of OFPNPG and imaged every 10 min over 70 min or 4 h, respectively. These samples were maintained at 37°C in a water bath between measurements.

3. Results

OFPNPG and OFPNP were easily distinguishable using ^{19}F CSI, as shown for two vials containing solutions of OFPNPG and OFPNP, respectively, with Na-TFA as internal chemical shift reference (Fig. 1). The conversion of OFPNPG to OFPNP by β-gal enzyme is shown in Fig. 2. Following addition of 80 U of β-gal enzyme to the left vial, conversion was detected by decrease in OFPNPG image intensity, which was accompanied by an increase in OFPNP image intensity. The OFPNPG intensity in the right vial (control) remained constant. Fig. 3 shows the conversion of OFPNPG to OFPNP by lacZ transfected rat 9L-glioma cells. Adding 10^8 9L-lacZ cells to a 70 mM solution of OFPNPG resulted in ~40% conversion to OFPNP over 70 min. Similar results were obtained with MCF-7-LacZ human breast cancer cells (Fig. 4). Over 4 h, 10^7 cells converted ~40% of OFPNPG to OFPNP.

4. Discussion

We previously demonstrated that OFPNPG and its analogues could be used to detect β-gal activity by NMR spectroscopy and identified OFPNPG as the best gene reporter molecule [13]. We now present a method to image β-gal activity in solution or in stably transfected cancer cells using ^{19}F CSI of OFPNPG. ^{19}F NMR provides a large chemical shift response to small changes in molecular structure or microenvironment [11]. Upon cleavage by β-gal, the substrate forms the aglycone OFPNP, which is shifted upfield by 4–6 ppm depending on pH [13]. Release of the pH-sensitive aglycones also suggests a novel approach to measuring pH at the site of enzyme activity. Rate of conversion in the presence of the enzyme found here was slower than our previous data since sample temperature was lower, in this case 18°C. The slower rate of conversion for the human breast cancer MCF7 cells compared to the glioma cells was due to lower cell number (by a factor of 10).

Although ^{19}F has a 100% natural abundance, signal/noise is of concern for any exogenously administered agent for in vivo imaging studies. We used a saturated solution of OFPNPG in PBS, but a further increase in solubility is possible by using aqueous DMSO. Trifluoromethyl analogues also provide a higher signal/noise, although the chemical shift response is much smaller

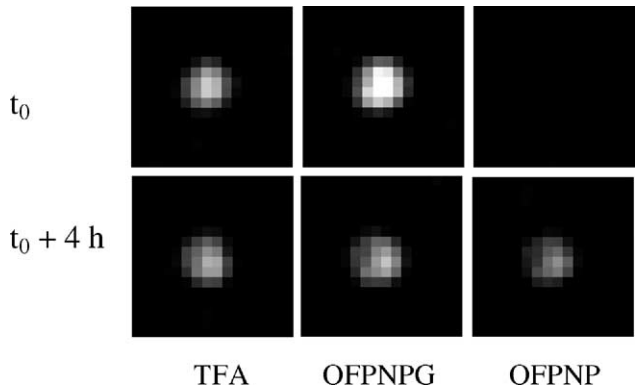

Fig. 4. Conversion of OFPNPG to OFPNP by MCF7-LacZ breast cancer cells detected using CSI. 10^7 cells were added to a vial containing OFPNPG (70 mM) and imaged over a period of 4 h.

($\Delta\delta = 1.5$ ppm) and may preclude effective imaging in vivo [14]. Other aglycones may also be introduced and we have shown that 6-fluoropyridoxol may be a less toxic substitute for nitrophenols [15]. OFPNPG specifically detected β-gal activity, but we note that ^{19}F NMR chemical shift response has been used by others to detect enzyme activity particularly with respect to pro-drug activation associated with gene-directed enzyme prodrug therapy. Others have examined fluorinated mustard drugs released by activity of glucuronidase [16] and carboxypeptidase G2 [17] and conversion of 5-fluorocytosine to 5-fluorouracil [6]. A major goal of our work was to seek minimally toxic gene reporter substrates and products, but we note that broad spectrum toxicity of nitrophenols could be applied to develop β-gal-activated chemotherapy using agents such as PFONPG.

We believe that noninvasive in vivo detection of gene reporter molecules will become increasingly important in biomedicine and it will be important to have diverse agents, genes and modalities for specific applications. Fluorophenyl β-D-galactosides offer a novel approach for determining β-gal activity. Key advantages of NMR reporters over radiolabeled substrates are the long shelf life, absence of radioactivity and the ability to distinguish between substrate and product. However, NMR does generally require millimolar reporter molecule concentrations, as opposed to micromolar (or lower) needed for optical and radionuclide approaches. The choice of appropriate probe and imaging modality depends critically on the nature of the problem at hand and an NMR approach could be suitable for many applications.

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